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Cyclodextrins and their Derivatives in Drug Delivery: A Review

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Abstract

The objective of the present review is to discuss the role and applications of cyclodextrins and their derivatives in drug delivery. Cyclodextrins are useful functional excipients that have enjoyed widespread attention and use. They are a family of cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity. Cyclodextrins have been used in the pharmaceutical industry as complexing agents to increase aqueous solubility of poorly soluble drugs, to increase stability, bioavailability and reduced drug irritation. Applications in the different drug delivery systems like oral, sublingual, buccal, ocular, nasal, transdermal, rectal, pulmonary, parenteral, colon-specific delivery, brain drug delivery, novel delivery systems like liposomes, nanoparticles, microparticles, in gene delivery and oligonucleotide delivery are discussed. Studies in both humans and animals have shown that cyclodextrins can be used to improve drug delivery from almost any type of drug formulation. However, addition of cyclodextrins to existing formulations without further optimization will seldom result in acceptable outcome. A number of cyclodextrin-based products have reached the market based on their ability to camouflage undesirable physicochemical properties.

Keywords: cyclodextrins, drug delivery, formulation, solubility, stability, bioavailability.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides consist of (α-1, 4)-linked α-D-glucopyranose units and due to the chair conformation of the glucopyranose units, the cyclodextrins are doughnut in shape rather than perfect cylinders. CDs are produced from starch by enzymatic conversion. As a result of their molecular structure and shape, they possess a unique ability to act as molecular containers by entrapping guest molecules in their internal cavity. The resulting inclusion complexes offer a number of potential advantages in pharmaceutical formulations. Cyclodextrins increase the water solubility of poorly soluble drugs to improve their bioavailability. Stability of the active pharmaceuticals in terms of oxidative, light and thermal stability can be improved through the formation of cyclodextrin inclusion complexes (1-3). Cyclodextrins have been using to reduce dermal, gastrointestinal or ocular irritation, mask unpleasant tastes or odors (4, 5), prevent adverse drug-excipient interactions and useful in converting the oils/liquids into powders to improve handling (6).

Structure, Derivatives, Properties of Cyclodextrins: Cyclodextrins are a general class of molecules composed of glucose units connected by α-1, 4 glycosidic linkages to form a series of oligosaccharide rings and contain a somewhat lipophilic central cavity and a hydrophilic outer surface. Due to the chair conformation of the glucopyranose units, the CDs

take the shape of a truncated cone or torus rather than a perfect cylinder. The hydroxyl functions are orientated to the cone exterior (which gives it a relatively hydrophilic character) with the primary hydroxyl groups of the sugar residues at the narrow edge of the cone and the secondary hydroxyl groups at the wider edge. The central cavity of the CD molecule is lined with skeletal carbons and ethereal oxygens of the glucose residue, which gives it a relatively lipophilic character (7, 8).

In nature, the enzymatic digestion of starch by cyclodextrin glycosyltransferase produces a mixture of cyclodextrins comprised of 6, 7 and 8 glucose units (α, β and γ cyclodextrin, respectively and were shown in Figure 1. Cyclodextrins are still commercially producing from starch by enzymatic digestion, specific enzymes are used to produce selective α, β or γ-cyclodextrin, as desired (9, 10). These three cyclodextrins are crystalline, homogeneous, non-hygroscopic in nature. The differing number of glucose units leads to slight differences in conformational structure, flexibility and size of the ring in terms of diameter (Fig. 1). While all three major cyclodextrins are water soluble, solubility is differs from one another, these differences result in higher exposure of hydrogen bonding hydroxyl groups to the aqueous environment and higher water solubility for γ and α–cyclodextrins than β-cyclodextrin (11).

Cyclodextrin derivatives can be prepared by chemical or enzymatic reactions. The main objective of such derivatizations may be: 1) to improve the solubility of the CD derivative and its inclusion complexes; 2) to improve the fitting and/or the association between the CD and its guest, with concomitant stabilization of the guest, reducing its reactivity and mobility; 3) to attach specific groups to the binding site (e.g., in enzyme modeling); 4) to form insoluble, immobilized CD-containing structures, polymers (e.g., for chromatographic purposes) (12).

Several chemical modifications techniques were applied cyclodextrins to obtain water-soluble cyclodextrin derivatives. Chemical modification of the hydroxyl groups, even by hydrophobic moieties such as methoxy functions present on the cyclodextrins, resulted in dramatic increase in their aqueous solubility. Later several

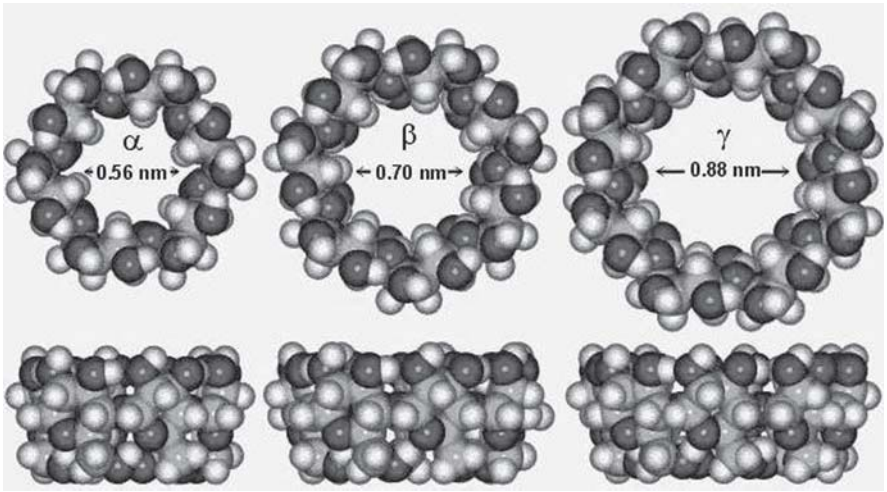

Fig. 1. Shape and size of α, β and γ-cyclodextrins

new derivatives of cyclodextrins for pharmaceutical interest include hydroxypropyl derivatives of β-CD and γ-CD (i.e., HP-β-CD and HP-γ-CD), the randomly methylated β-CD (RM-β-CD), sulfobutylether β-CD (SBE β-CD), Monochlorotriazinyl beta cyclodextrin (MCT-β-CD), Heptakis-β-CD [Heptakis (2-x-amino-O-oligo(ethylene oxide)-6-hexylthio) beta cyclodextrin] and the so called branched CDs such as maltosyl-β-CD (G2-β-CD) etc., came available (13). The main reason for the solubility enhancement in the alkyl derivatives is that chemical manipulation transforms the crystalline α, β or γ-cyclodextrins into amorphous mixtures of isomeric derivatives (7, 14). Properties of natural cyclodextrins and some of their derivatives such as average number of substituents per glucopyranose repeat unit, molecular weight, solubility are shown in Table 1.

Cyclodextrin molecules are relatively high molecular weight ranging from 1000 to 2000 Da with a large number of hydrogen donors and acceptors, and are consequently poorly absorbed through biological membrane. Natural α- and β-cyclodextrins cannot hydrolyze by human salivary and pancreatic amylases, whereas ã-cyclodextrin can hydrolyze by human salivary and pancreatic amylases, but all three are subjected to fermentation by the intestinal microflora. Hydrophilic cyclodextrins are non-toxic in nature at low to moderate concentrations in oral dosage forms (15, 16). The natural cyclodextrins and their derivatives are used in topical and oral dosage forms, but only γ-cyclodextrin and the hydrophilic derivatives of β- and γ-cyclodextrin can be used in parenteral formulations. γ-Cyclodextrin forms visible aggregates in aqueous solutions and, thus, is not well suited for parenteral formulations (17). β-cyclodextrin cannot be used in the parenteral formulations because of its nephrotoxicity. Lipophilic cyclodextrin derivatives, such as methylated cyclodextrins, are to some extent absorbed from the gastrointestinal tract into the systemic circulation and have been shown to be toxic after parenteral administration (15). Presently, oral administration of methylated β-cyclodextrin is limited by its potential toxicity.

Inclusion complex formation: Cyclodextrins are able to form solid inclusion complexes with active drug moieties (host–guest complexes) with a very wide range of solid, liquid and gaseous compounds by a molecular complexation. In these cyclodextrin inclusion complexes, a guest molecule is held within the cavity of the

Table 1. Natural cyclodextrins and their derivatives that can be found in marketed pharmaceutical products.

Cyclodextrin	Substitution[a]	MW[b]	Solubility inwater (mg/mL)[c]
α-Cyclodextrin	–	972	14.5
β-Cyclodextrin	–	1135	1.85
2-Hydroxypropyl-β-cyclodextrin	0.65	1400	>600
Randomly methylated β-cyclodextrin	1.8	1312	>500
β-Cyclodextrin sulfobutyl ether sodium salt	0.9	2163	>500
γ -Cyclodextrin	–	1297	23.3
2-Hydroxypropyl- γ -cyclodextrin	0.6	1576	>500

[a] Average number of substituents per glucopyranose repeat unit.
[b] MW (Molecular Weight) in Daltons.
[c] Solubility in pure water at approx. 25°C.

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cyclodextrin host molecule. Complex formation is a dimensional fit between the host cavity and guest molecule was represented in Fig. 2 (18). The lipophilic inner cavity of the cyclodextrin molecules provide a lipohilic microenvironment, into which appropriately sized non-polar moieties of the guest active moieties can enter to form inclusion complexes (19). No covalent bonds are formed or broken during formation of the inclusion complex (20). Release of enthalpy-rich water molecules from the cavity is the main driving force for complex formation, electrostatic interaction, van der Waals interaction, hydrophobic interaction, hydrogen bonding, release of conformational strain, and charge-transfer interaction. All these forces are relatively weak, allowing free drug molecules in solution to be in rapid equilibrium with drug molecules bound within the cyclodextrin cavity. Water molecules are displaced by more hydrophobic guest molecules present in the solution to attain an apolar-apolar association and decrease of cyclodextrin ring strain resulting in a more stable lower energy state.

The inclusion of guest molecules within the host cyclodextrin is in dynamic equilibrium and not a fixed or permanent. Binding strength depends on how well the 'host–guest' complex fits together and on specific local interactions between surface atoms. Several techniques are used to form CD inclusion complexes, like co-precipitation, slurry complexation, paste complexation, damp mixing, heating method, extrusion and dry mixing.

Phase Solubility studies: Phase Solubility studies were first described by Higuchi and Connons in 1964 (21, 22) in their pioneering research work. In phase solubility studies the effect of complexing agents on the compound being solubilized is to determine not only the value of the stability constant but also to give stoichiometry of the equilibrium. Experimentally phase solubility studies were conducted by

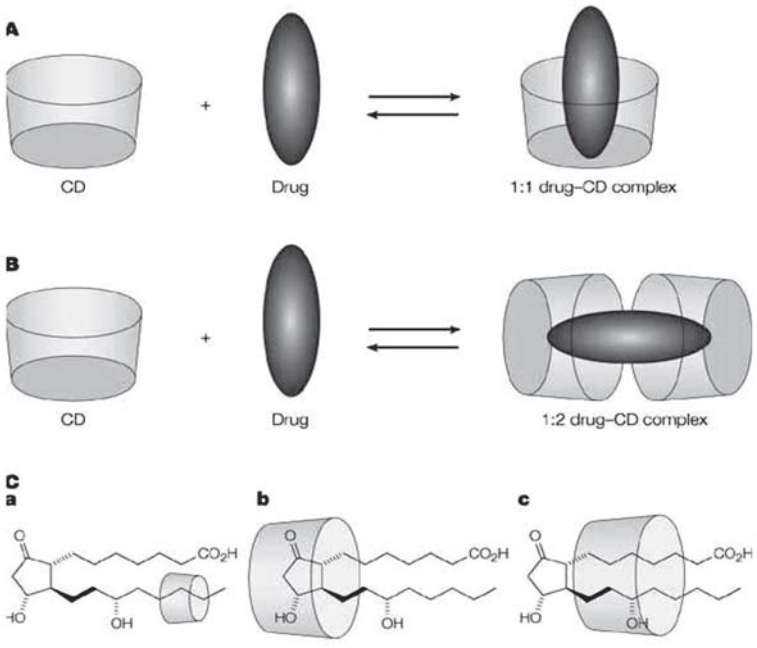

Fig.2. Illustration of cyclodextrin inclusion complex formation

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adding, an excess of a poorly water-soluble drug into several vials to which a constant volume of an aqueous vehicle containing successively larger concentrations of the cyclodextrins/ cyclodextrin derivatives are added. The need for excess drug is based on the desired to maintain as high a thermodynamic activity of the drug as possible. The vials are shaken at constant temperature until equilibrium is established. The suspensions are then filtered and the total concentration of the drug determined based on appropriate analytical techniques (UV Visible Spectrophotometer or HPLC). The Phase Solubility profile is then constructed by assessing the effect of the cyclodextrin on the apparent solubility of the drug. Based on the shape of the generated Phase Solubility relationships, several types of behaviors can be identified (23) Phase Solubility diagrams fall into two major types: Type A profile and Type B profile (Fig. 3).

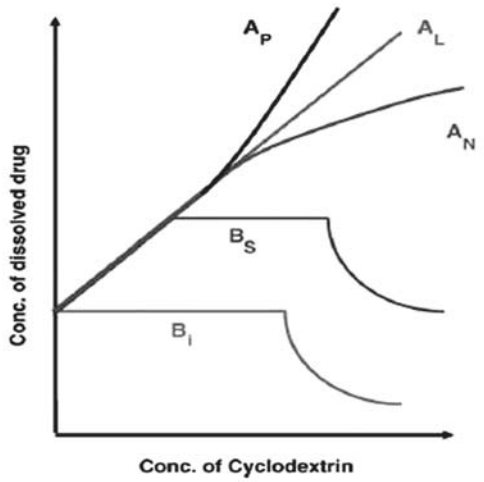

Fig. 3. Phase Solubility diagram represents A and B type profiles

A-type phase solubility profiles: In A type solubility profile, the apparent solubility of the drug or substrate increase as a function of cyclodextrin concentration. There are three subtype A phase solubility profiles have been defined: A_L type profiles indicate a linear increase in solubility as a function of solubilizer/cyclodextrin concentration, A_P systems indicate an isotherm wherein the curve deviates in a positive direction from linearity (i.e. the solubilizer/cyclodextrin is proportionally more effective at higher concentrations) and A_N relationships indicate a negative deviation from linearity (i.e. the cyclodextrin is proportionally less effective at higher concentrations). Taken as a whole, these isotherms indicate that water soluble complexes are being formed with solubilities higher than that of the uncomplexed substrate. Generally A_L-type relationships follows first order with respect to the cyclodextrin and may be first or higher order with respect to the drug. If the slope of the A_L isotherm is greater less than one, first order complexes are assumed to be involved in the solubilization and slope value is higher than one indicates higher order complexes are formed.

B-type phase solubility profiles: In B type phase solubility profiles are indicative of the formation of complexes with limited water solubility and are generally observed with naturally occurring cyclodextrins, especially with β-CD. Two subclasses have been described in B-type profile including B_S and B_I systems. B_S-type isotherms denote complexes of limited solubility and a B_I-curve are indicative of insoluble complexes.

Formulation and process variables influencing the cyclodextrin inclusion complexes

Type of Cyclodextrin: Type of cyclodextrin can influence the inclusion complex formation as well as the performance of drug/CD complexes. For better inclusion complexation, the cavity size of cyclodextrin should be suitable to accommodate a drug molecule of particular size. Nagase Y, 2001 *et al.,* (110) reported that inclusion complexation can be better when the cyclodextrin and the drug carry opposite charge but may decrease when they carry the same charge in comparison with neutral cyclodextrins. For many acidic drugs forming anions, the cationic (2-hydroxy-3-[trimethylammonio] propyl)-β-CD

acted as an excellent solubilizer in comparison to neutral and ionic cyclodextrins. In the case of ionisable drugs, the presence of charge may play a significant role in drug/CD complexation and hence a change in the solution pH can vary the complex constant. In general, ionic forms of drugs are weaker complex forming agents than their nonionic forms, but in the case of mebendazole, the un-ionized form was less included in HP-β-CD than the cationic form (24). Arias-Blanco MJA, 1998 et al., (111) reported that the cavity size of β-CD was suitable for complexation of gliclazide while that of α-CD was insufficient to include gliclazide into cyclodextrin ring.

Process temperature: Formulation and process temperature changes can affect drug/cyclodextrin complexation. In general most of the cases, increasing in the temperature decreasing the apparent stability constant of the inclusion complex and the effect was reported to be a result of possible reduction of drug/cyclodextrin inclusion complex interaction forces, such as van der Waals and hydrophobic forces with increasing in temperature. However, temperature changes may have negligible effect when the drug/cyclodextrin interaction is predominantly entropy driven (i.e., resulting from the liberation of water molecules hydrated around the charges of guest and host molecules through inclusion complexation).

Method of preparation: Method of preparation, viz physical mixing/co-grinding, kneading, solvent evaporation, co-precipitation, spray drying, or freeze drying can affect drug-cyclodextrin complexation. The effectiveness of a method depends on the nature of the drug and cyclodextrin used in the preparation (25, 26). Most of the cases, spray drying and freeze drying were found to be most effective for drug, cyclodextrin inclusion complexation, resulting to improvement of solubility, stability and bioavailability. In spray drying and freeze drying amorphousization of the drug taking place during complexation. In case of tolbutamide:β-CD inclusion complexation method of preparation showed no influence on the dissolution performance.

Water-soluble polymers or ion pairing agents added in small amounts, enhanced cyclodextrin solubilizing effect by increasing the apparent complex stability constant. The polymers or ion pairing agents due to their direct participation in drug complexation, improve both pharmaceutical and biological properties of drug/cyclodextrin complexes. Some of the inactive ingredients/additives may compete with drug molecules for cyclodextrin cavities and thus decrease the apparent complex stability constant. Water structure forming agents when added to cyclodextrin solutions generally increase the total drug solubility, but they showed opposite effects with clotrimazole. Simultaneous complexation and salt formation with hydroxy carboxylic acid (HA) significantly increased the cyclodextrin solubilizing power for a sparingly water-soluble drug by forming drug/CD/HA multicomponent/ternary complexation systems. Co-solvents can improve the solubilizing and stabilizing effects of CDs, e.g., use of 10% propylene glycol in development of an oral itraconazole preparation containing 40% of HP-β-CD. Sometimes co-solvents may hinder drug complexation by competitive inclusion, e.g., presence of 10% propylene glycol decreased the solubilizing effect of HP-β-CD for itraconazole. On dilution, the presence of propylene glycol favored absorption and precipitation of itraconazole in GI fluids and formulation by providing increased percentage of the free drug. The increased percentage of the free drug in presence of co-solvent was reported to be a result of lesser intrinsic solubility of the drug compared with the dilution concentration line at a given HP-β-CD concentration (27).

Role of Cyclodextrin on drug solubility, dissolution rate and stability: Cyclodextrins (CDs) have been playing an important role in development of poorly aqueous-soluble drugs by improving drug solubility and dissolution rate through inclusion complexation. Reduction of

drug crystallinity on complexation or solid dispersion with CDs also contributes to the cyclodextrin increased apparent drug solubility and dissolution rate. CDs, as a result of their ability to form in situ inclusion complexes in dissolution medium, can enhance drug dissolution even when there is no complexation in the solid state.

CDs can improve the stability of several labile drugs by preventing hydrolysis, oxidation, dehydration, and photodecomposition of the formulation. Role of Cyclodextrin and its derivatives on drug solubility, dissolution rate and stability were represented in Table 2.

Safety and biocompatibility of cyclodextrins and its derivatives: The chemical structure of cyclodextrins (i.e., the large number of hydrogen donors and acceptors), their molecular weight is more than 972 Da and their very low partition coefficient (approximately log *P*o/w between less than 3 and 0) are all characteristics of compounds that do not readily permeate biological membranes. Studies have shown that only negligible amounts of hydrophilic cyclodextrins and drug/cyclodextrin complexes are able to permeate lipophilic membranes such as gastrointestinal mucosa and skin. All toxicity studies have demonstrated that when administered orally cyclodextrins are practically non-toxic due to lack of absorption from the gastrointestinal tract. However, the lipophilic methylated β-cyclodextrins are surface active and they are to some extent (~10%) absorbed from the gastrointestinal tract and consequently only limited amounts of these lipophilic cyclodextrin derivatives can be included in oral formulations, and they are unsuited for parenteral formulations. Due to toxicological considerations β-cyclodextrin cannot be used in parenteral formulations and

Table 2. Role of cyclodextrin and their derivatives on drug solubility, dissolution rate and stability

Cyclodextrins	**Examples of CD-enhanced Solubility and Dissolution**
α-Cyclodextrin	Praziquantel
β-Cyclodextrin	Piroxicam, Nimesulide, Lorazepam, Ketoprofen, Praziquantel, Chlorthalidone, Itraconazole, Ibuprofen, Griseofulvin
Dimethyl-β-cyclodextrin (DM-β-CD)	Naproxen, Camptothesin
Random methyl-β-cyclodextrin	Tacrolimus
Hydroxypropyl-β-cyclodextrin (HP-β-CD)	Griseofulvin, Albendazole, Levemopamil HCl, Sulfomethiazole, Itraconazole, Ketoprofen, Carbamazepine, Zolpidem
γ-CD	Omeprazole, Digoxin, Praziquantel
Cyclodextrins	**Examples of CD-enhanced Stability**
β-Cyclodextrin	Glibenclamide, Diclofenac sodium, Flutamide, Atorvastatin Calcium
Hydroxypropyl-β-cyclodextrin (HP-β-CD)	Promethazine, Quinaril, Doxorubicin, Rutin, Paclitaxel, Spiranolactone,
SBE -β-CD	Spiranolactone, Melphalan and Carmustine
γ-CD	Digoxin

the usage of α-cyclodextrin in parenteral formulations is severely limited although it can already be found in one marketed formulation. In animal studies, β-cyclodextrin has been found to be virtually non-toxic when given intravenously. Extensive toxicological studies have been completed for 2-hydroxypropyl-β-cyclodextrin as well as for sulfobutylether β-cyclodextrin, both of which can be found in marketed parenteral formulations at relatively high concentrations.

Regulatory status of cyclodextrins: Cyclodextrin monographs can be found in several pharmacopoeial sources. Their regulatory status continues to evolve (28, 29). β-CD is listed in a number of pharmacopoeial sources including the US Pharmacopoeia/National Formulary (USP/NF), European Pharmacopoeia (Ph.Eur.) and Japanese Pharmaceutical Codex (JPC). α-CD is similarly listed in the Ph.Eur., USP/NF and JPC and β-CD is referenced in the JPC and will soon be included in the Ph.Eur., and USP/NF. A monograph for HP-β-CD is available in the Ph.Eur., and a draft has been circulated for the USP/NF. Other derivatives are not yet compendial but efforts are underway for their inclusion. β-CD, β-CD and α-CD were also introduced into the generally regarded as safe (GRAS) list of the FDA for use as a food additive in 2004, 2001 and 2000, respectively, HP-β-CD and SBE β-CD are cited in the FDA's list of Inactive Pharmaceutical Ingredients.

Cyclodextrins in drug delivery: Cyclodextrins are currently used in the pharmaceuticals to improve the aqueous solubility, stability and bioavailability of poorly aqueous soluble and unstable drugs (7). Cyclodextrins will form hydrophilic inclusion complexes with lipophilic active moieties. In aqueous solutions drug molecules bound with the cyclodextrin in the inclusion complex are in a dynamic equilibrium with the free drug molecule. Thus, cyclodextrins enhance the aqueous solubility of drugs without changing their intrinsic ability to permeate lipophilic membranes (30). Due to their size and hydrophilicity insignificant amounts of cyclodextrins and drug/cyclodextrin complexes are able to penetrate into lipophilic biomembranes, such as intact skin. In general cyclodextrins enhance drug delivery through biomembranes by increasing the drug availability at the membrane surface. At the surface the drug molecules partition from the cyclodextrin cavity into the lipophilic membrane (31, 32). Thus, properly designed cyclodextrin formulation will increase the drug concentration gradient over the membrane, which will increase the drug flux through the membrane. Since drug/cyclodextrin complexes do not readily permeate biomembrane, excess cyclodextrin in pharmaceutical formulations can reduce drug bioavailability. Cyclodextrins are used in almost all drug delivery systems and few important delivery systems were described in the subsequent sections.

Oral Drug Delivery: The effect of cyclodextrins on oral drug absorption can be explained in the context of the Biopharmaceutics Classification System (BCS) (28). BCS Class I drugs have good aqueous solubility and permeate easily through the aqueous diffusion layer and possess sufficient lipophilicity to permeate through the gastrointestinal mucosa. In general, hydrophilic cyclodextrins are not able to improve the bioavailability of Class I drugs. However, cyclodextrin can be used to reduce local drug irritation, taste masking and increase rate of drug absorption. BCS Class II drugs have limited aqueous solubility and good permeability, resulting in dissolution-rate limited oral absorption. Thus, permeation through the aqueous diffusion layer adjacent to the mucosal surface will also be slow due to their low aqueous solubility. Water-soluble cyclodextrin complexes of these drugs will enhance their diffusion to the mucosal surface leading to improved oral bioavailability. BCS Class III drugs are water soluble, but do not easily permeate biological membranes due to their size and/or extent of hydration. Consequently, formation of hydrophilic drug/cyclodextrin complexes will not enhance their oral bioavailability, but will, if anything, reduce the ability of dissolved drug molecules to

partition from the aqueous exterior into the gastrointestinal mucosa. BCS Class IV drugs are water insoluble and do not readily permeate through biological membranes. These can, for example, be water-insoluble zwitterions or relatively large lipophilic molecules. Hydrophilic water-insoluble compounds such as zwitterions do not readily form cyclodextrin complexes and, thus, hydrophilic cyclodextrins are not likely to improve their oral bioavailability. However, cyclodextrins are capable of increase in aqueous solubility of some large lipophilic molecules leading to increased drug availability at the mucosal membrane. This will frequently lead to increased oral bioavailability. Modification of the drug release site and/or time profile by cyclodextrins is shown in Table 3.

CDs in Oral Immediate release dosage forms: CDs have been extensively used to improve the aqueous solubility and the oral bioavailability of poorly aqueous soluble actives such as cardiac glycosides, antiepileptics, benzodiazepines, antidiabetics, vasodilators etc. (33). These improvements are mainly attributed to the increase in solubility and wettability of drugs through the formation of inclusion complexes. Complexation of β-CD with imidazole antifungal agents, such as ketoconazole and econazole, provides increased solubility and enhanced bioavailability (34, 35). Cyclodextrins and their derivatives such as HP-β-CD and randomly methylated β-CDs are utilized in the improvement of solubility, stability and bioavailability of Atorvastatin calcium (36, 37). Chinna Reddy *et al* reported that the solubility, stability and bioavailability of atorvastatin calcium were significantly improved due to inclusion complexation with cyclodextrins in a 1:1 stoichimetric ratio (38). The stabilizing effect of CDs on unstable drugs is also responsible for the improvement of oral bioavailability. Uekama *et al*., reported that the γ-CD complex decreases acid hydrolysis of cardiac glycosides and thus improves the oral absorption and bioavailability of digoxin in dogs (38). Highly hydrophilic CD derivatives, such as HP-β-CD (39), maltosyl-β-CD (40) and SBE-β-CD sulfate and sulfobutylether-β-CD; (41) have been used to obtain an immediate release formulation that is readily dissolved in GIT, enhancing the oral bioavailability of poorly water-soluble drugs.

CDs in Delayed release dosage forms: Horikawa *et al.* studied the release of the water soluble drug molsidomine, is an orally active, long acting vasodilating agent from the tablets of CME-β-CD complex using male beagle dogs with controlled gastric acidity. Under high gastric acidity, molsidomine absorption was significantly

Table 3. Modification of the Drug Release Site and/or Time Profile by Cyclodextrins

Release Pattern	Aim	Use of Cyclodextrin
Immediate release	Enhanced dissolution and absorption of poorly water soluble drugs	HP-β-CD, Dimethyl-β-CD (DM-β–CD), SBE-β-CD and branched-β-CDs
Prolonged release	Sustained release of water-soluble drugs	Ethylated β-CDs, acylated β-CDs
Modified release	More balanced oral bioavailability with prolonged therapeutic effects	Simultaneous use of different CDs and/or other excipients
Delayed, pH-dependent release	(Enteric) Acid protection of drugs	Carboxymethyl ethyl- β-CD (CME-β-CD)
Site-specific release	Colon-targeting	Drug/CD conjugate

retarded compared to low gastric acidity conditions. The delayed absorption effect under high gastric acidity was more pronounced under fasted conditions (42).

Uekama *et al.,* studied on diltiazem HCl, a water-soluble drug for delayed release formulation. Diltiazem HCl was complexed with CME-β-CD and compressed into a tablet. The drug release rate was quite slow in the low-pH solutions and increased with an increase in pH. The release of water-soluble diltiazem HCl from CME-β-CD was suppressed at a lower pH because of ionization of the carboxyl group. These studies indicated that the release rate of water-soluble drugs can be suppressed in the low-pH region in the stomach and increased at intestinal pH values because of the ionization of the carboxyl group of the drug molecule, thereby showing suitability for drug targeting to specific areas in the intestine (43).

Tiaprofenic acid (TA), an NSAID, is associated with gastrointestinal toxicity and erratic bioavailability. Tiaprofenic acid inclusion complex was prepared by kneading method using DE-β-CD in a 1:1 molar ratio. The dissolution profile of TA as a powder was tested at pH values of 1.5, 3.0, 4.5, 6.8 and 7.4. The in vitro dissolution rate from the TA/DE-β-CD complex increased by increasing the pH value and complete release was observed at a pH of 7.4. In vivo studies in male Sprague Dawley rats administration of solid particles of complexes by means of gastric intubations followed by ingestion of 500µL of water. The studies showed a prolonged *T*max, which most likely resulted from slow or no release of the drug in the stomach and duodenum, both of which have low pH ranges. The drug was released and was absorbed immediately and completely in the distal intestine, which has an alkaline pH (44).

CDs in Modified release dosage forms: Nifedipine has low oral bioavailability due to its poor aqueous solubility; hence the release rate of nifedipine must be modified in order to obtain a more balanced oral bioavailability with therapeutic effect. Wang *et al.,* (45) developed a double-layer tablet employing an amorphous nifedipine inclusion complex prepared by spray drying method with HP-β-CD and HCO-60® (non-ionic detergent) as the fast release portion to attain initial rapid dissolution and hydroxypropylcelluloses (HPCs) with different viscosity grades (low, medium and high) as a slow-release portion to attain delayed dissolution. Recently, Okimoto *et al.,* (46) developed a novel osmotic pump tablet for prednisolone using (SBE) 7m-β-CD, which acts as a solubilizer and an osmotic agent. Cyclodextrins containing marketed pharmaceutical formulations are shown in Table 4.

Sublingual and buccal drug delivery: Sublingual drug delivery is one of the most efficient methods to bypass hepatic first-pass metabolism. In sublingual method the drug dissolves in the mucosa and then enters in to the systemic circulation. Rapidly dissolving complexes of drugs and CDs are well suited for sublingual or buccal administrations, which avoid the hepatic first-pass metabolism. However, in order to enter into the systemic circulation the drug must dissolve in the saliva. Due to the small volume of saliva in the mouth, the therapeutic dose has to be relatively small and usually dissolution enhancers must be included in the formulation. In sublingual formulations the complexation of poorly water-soluble drugs such as 17β-estradiol (47), androstenediol (48), clomipramine (49) and danazol (50) with cyclodextrins has been shown to increase in solubility, permeability and bioavailability. However, the interactions between cyclodextrins and sublingual mucosa (i.e., cyclodextrins acting as conventional penetration enhancers) cannot be excluded. Results from *in vivo* absorption studies showed that sublingual administration of randomly methylated β-CD (RM-β-CD) containing Δ^9-tetrahydrocannabinol (THC) formulation increases the bioavailability of THC compared with oral administration (51).

Jug, M *et al.,* (52) reported that the Influence of hydroxypropyl-β-cyclodextrin complexation on

Table 4. Marketed pharmaceutical products containing cyclodextrins

Drug/cyclodextrin	Trade name	Formulation	Country
α-Cyclodextrin			
Alprostadil (PGE1)	Prostavastin, Rigidur	I.V. solution	Japan, Europe, USA
OP-1206	Opalmon	Tablet	Japan
Cefotiamhexetil HCl	Pansporin T	Tablet	Japan
β-Cyclodextrin			
Benexate HCl	Ulgut, Lonmiel	Capsule	Japan
Cephalosporin(ME1207)	Meiact	Tablet	Japan
Chlordiazepoxide	Transillium	Tablet	Argentina
Dexamethasone	Glymesason	Ointment	Japan
Diphenhydramin HCl	Stada-Travel	Chewing tablet	Europe
Nicotine	Nicorette, Nicogum	chewing gum	Europe
Nimesulide	Nimedex	Tablet	Europe
Nitroglycerin	Nitropen	Sublingual tablet	Japan
2-Hydroxypropyl-β CD			
Cisapride	Propulsid	Suppository	Europe
Itraconazole	Sporanox	Oral and I.V. solutions	Europe, USA
Mitomycin	Mitozytrex	I.V. infusion	Europe, USA
Methylated β-CD			
Chloramphenicol	Clorocil	Eye drop solution	Eye drop solution
17â-Estradiol	Aerodiol	Nasal spray	Europe
Sulfobutylether β-CD			
Voriconazole	Vfend	I.V. solutionl	Europe, USA
Ziprasidone mesylate	Geodon, Zeldox	M solution	Europe, USA
2-Hydroxypropyl-γ-CD			
Diclofenac sodium	Voltaren	Eye drop solution	Europe
Tc-99 Teoboroxime	Cardiotec	I.V. solution	USA

piroxicam release from buccoadhesive tablets. In their research they found that HP-β-CD can enhance the release rate of piroxicam from tableted matrices formulated with swellable hydrophilic polymers, hydroxypropylmethyl cellulose (HPMC) and Carbopol 940 (C940). The increase in the release rate of piroxicam from buccal tablets could be attributed to the ability of HP-β-CD to form a complex with piroxicam, resulting in an increase of apparent drug solubility. Higher medium penetration rate into the complex containing tablets may also contribute to the improved release rate. Piroxicam was released from the polymer matrices in a constant mode over the passage of time, thus providing a prolonged effect.

Cappello, B *et al.,* (53) studied the role of HP-β-CD on carvedilol containing poly ethylene oxide (PEO) tablets for buccal delivery system. The amount of carvedilol permeated from PEO tablet was higher in the case of HP-β-CD containing tablets compared to PEO tablets containing only carvedilol. Cyclodextrins are responsible for an increase in the erosion rate of the buccal tablet resulting in an improvement in the dissolution rate of the drug inside the

polymeric matrix. The erosion effect is the crucial factor, which determines the increase of release rate from the tablets in solution. They observed a twenty-fold increase in the amount of carvedilol permeated through porcine buccal mucosa in comparison with absence of cyclodextrin in the formulation .

Chinna Reddy *et al.,* (54) studied the release of felodipine from buccal tablets comprising HP-β-CD - felodipine complex and hydroxylpropyl methyl cellulose and reported a complete and sustained release of the drug associated with an enhanced buccal permeation. These results could be attributed to the ability of HP-β-CD to form a complex with felodipine, resulting in an increase of apparent drug solubility, dissolution rate and permeability .

Ocular Delivery: In ocular delivery drug administration in the form of topically applied low viscosity aqueous eye drops. Ophthalmic irritation is a common drawback in ophthalmic drug development and in their clinical use. CDs are generally included in the ocular formulations to decrease the irritation effect of ophthalmic drugs by forming inclusion complexes.

HP-β-CD is the most commonly used CDs in aqueous eye drop formulations (55). Numerous studies in animals as well as in human beings have shown that HP-β-CD is well tolerated in aqueous solutions, even at high concentrations as much as 45%. Application of one drop of aqueous eye drop solution containing 18% HP-β-CD to humans, three times a day for 28 days, was well tolerated in the eye (56). Jarho, P et al., studied the effect of SBE7-β-CD on pilocarpine prodrug in rabbits and reported that SBE7-β-CD improved the ocular delivery and tolerability of pilocarpine in aqueous solutions (57). Other CDs and CD derivatives which might be considered safe upon topical administration in aqueous eye drop solutions include maltosyl β-CD, γ-CD and hydroxypropyl- β-cyclodextrin (HP-γ-CD), and, at low concentrations, α-CD and RM- β-CD.

Irritation of pilocarpine in the eye is due to the rapid absorption of the pilocarpine prodrug into the lipophilic corneal epithelium and/or precipitation of prodrug molecules in the pre-corneal area. Pilocarpine/SBE-β-CD inclusion complexes can be considered to be act as a depot in the precorneal area that limits the free prodrug concentration in that area to a non-irritating level (58). The major ocular drug administration is usually aqueous solutions in which most of the drugs are prone to chemical degradation. One of the most common pharmaceutical advantages of CDs is to increase the drug stability in aqueous solutions. Lee, V.H.L *et al.,* studied on dipivefrine, a prodrug of epinephrine, has currently replaced epinephrine in the treatment of glaucoma. The main drawback of dipivefrine is its low aqueous stability. Dipivefrine is stable at a pH range of 2.5 to 3.5, so the stability problem has been overcome by formulating a dipivefrine solution in this pH range. Lee, *et al.,* reported that inclusion of SBE-β-CD in dipivefrine aqueous ocular formulation at pH 5 and pH 7.4, the negatively charged SBE-β-CD increases the aqueous stability of positively charged dipivefrine 15-30 times and 20-200 times, respectively (59). Cyclodextrin based dexamethasone eye drops are well tolerated in the eye and seem to provide higher bioavailability and clinical efficiency than presently available steroid eye drop formulations (60).

Nasal Drug Delivery: The nasal route is one of the effective ways to bypass the hepatic first-pass metabolism. Drugs of highly lipophilic in nature are difficult to deliver through the nasal route due to their poor aqueous solubility. High molecular weight hydrophilic drugs like peptides and proteins show poor nasal absorption. Administration of lipophilic drugs such as steroidal hormones estradiol and progesterone along with CDs has shown rapid absorption, this may be due to formation of inclusion complexation with cyclodextrins (61). The estradiol nasal spray Aerodiol® (Servier) represents the successful use of cyclodextrins in nasal applications; each spray delivers 70 μL of solution, which contains 150 μg of estradiol dissolved in aqueous RM-β-CD solution.

Dimethyl-β-CD (DM-β–CD) and HP-β-CD have shown an enhanced and sustained level of morphine in the plasma and cerebrospinal fluid (62). CDs have been shown to enhance the concentration and improve the stability of the antimigraine drug dihydroergotamine (63) and increased nasal absorption of oligopeptide drugs like buserelin (64) and leuprolide (65). Calcitonin, containing a 5% concentration of methylated-β-CD, nasal absorption significantly increased compared to intravenous or subcutaneous administration of the drug (66). Glucagons (67) and insulin (68) nasal bioavailability was significantly improved with incorporation of dimethyl-β-CD (DM-β-CD) in the formulation. It can be concluded from the various studies reported in the literature that methylated β–CD derivatives, such as DM-β-CD significantly enhances the nasal absorption of peptides and proteins. In addition, the local toxicity of dimethyl-β-cyclodextrin, indicated by ciliary beat frequency, has been shown to be very mild compared with other absorption-enhancing agents and preservatives (e.g., benzalkonium chloride) used in nasal formulations (69).

Transdermal drug delivery: Drugs have been delivered by the transdermal route for both local and systemic action. As the drug enters systematic circulation directly, the first pass effect is eliminated; it also eliminates the factors that influence gut absorption. The transdermal drug transport is greatly limited by stratum corneum permeation characteristics; so attempts at improving topical absorption have been reported. Cyclodextrins enhance drug delivery through aqueous diffusion layers (i.e., aqueous diffusion barriers), but not through lipophilic barriers such as the stratum corneum. If the drug release is from an aqueous-based vehicle or if an aqueous diffusion layer at the outer surface of the skin is a rate-determining factor in dermal drug delivery, then cyclodextrins can act as penetration enhancers. However, if drug penetration through the lipophilic stratum corneum is the main rate-determining factor then cyclodextrins are unable to enhance the delivery (54). Through cyclodextrin complexation it is possible to enhance significantly hydrocortisone delivery from cream formulations to the skin (70).

Dermal corticoids like betamethasone (71) and beclamethasone dipropionate (72) showed enhanced release from hydrophilic ointment bases after complexation with β-CD and/or γ-CD. Vehicle types used markedly affect the enhancing effect of CDs on the drug release, e.g., prednisolone complexation with DM-β-CD in non-aqueous ointment bases such as macrogol decreases the release. These decreasing effects of the hydrophilic CDs may be due to the lowering of the drug solubility via the complex formulation (73). CDs are also able to enhance the dermal delivery of NSAIDs. Lin *et al.,* (74) reported that the anti-inflammatory effects of indomethacin in hydroxyethylcellulose hydrogels, a hydrophilic base, were significantly enhanced by complexation with β-CD and HP-β-CD in healthy volunteers. This can be due to permeation enhancement of lipophilic drugs such as corticosteroids and NSAIDs through the skin by increasing the drug thermodynamic activity in water containing vehicles.

Recently, CDs in dermal delivery of proteins and peptides has been noted. For example, a combination of β-CD and the permeation enhancer Azone achieved higher percutaneous absorption of a peptide drug, nafarelin acetate, and a luteinizing hormone releasing analog (75).

Cyclodextrins have also been used to reduce permeability of compounds into skin. For example, addition of an excess of HP-β-CD to a vehicle containing the UV-absorbing compound oxybenzone (a common sunscreen) (more than needed to solubilizing the compound) reduced significantly transdermal permeation of the compound, thus preventing permeation of the sunscreen into skin (76, 77).

Studies show that at higher concentrations parent CDs and chemically modified CDs caused skin irritation in guinea pigs in the order γ-CD < α-CD < β-CD; this result largely depends on their

abilities to extract lipids from stratum corneum (78). As regards CD derivatives, DM-β-CD is known to extract the components from stratum corneum, which leads to skin irritation.

Numerous studies have shown that excess cyclodextrins do, like in the case of ophthalmic drug delivery, decrease drug delivery through excised skin (30). Use of cyclodextrins and their derivatives in transdermal drug delivery are represented in Table-5.

Rectal Drug Delivery: Rectal drug delivery is an alternative route of drug administration for patients who have difficulties in swallowing, suffering from nausea or vomiting, for infants or children and old people intended for systemic use. However, rectal delivery is limited by the low media volume, limited absorbing surface area and drug degradation by microorganisms present in the rectum resulting low bioavailability. CDs are useful in rectal delivery to improve the drug release, stability, bioavailability and alleviation of local irritation (79). Enhancement of rectal absorption of lipophilic drugs is based on the improvement of drug release from vehicles and the dissolution rates in rectal fluid. CDs directly act on the rectal epithelial cells in case of unabsorbable drugs like antibiotics, peptides and proteins. Drug-cyclodextrin inclusion complexes improve the chemical stability of the drugs in suppository bases and reduce the drugs bioconversion to pharmacological inactive metabolites in the rectum. For example, AD-1590, an acidic NSAID, with β-CD prevents its auto-oxidation (80). These stabilizing effects of CDs are attributed to insolubilization of the drugs in the lipophilic suppository base. CDs have been reported as co-enhancers. Watanabe *et al.,* (81, 82) reported that CDs enhanced the permeability of proteins such as insulin and recombinant human granulocyte colony stimulating factor through the rectal epithelial cells of rabbits. CDs reduce gastrointestinal mucosal irritation, CDs have also been reported to reduce rectal irritation caused by NSAIDs. For example, HP-β-CD significantly reduced the irritation of rectal mucosa caused by Biphenyl acetic acid (BPAA) and ethyl-4-biphenylyl acetate (EBA) administration of lipophilic suppositories to rats (83, 84). Use of cyclodextrins and their derivatives in rectal drug delivery are represented in Table-5.

Pulmonary drug delivery: Pulmonary administration of drugs is to treat asthma, chronic obstructive pulmonary disease or other lung diseases (85). Pulmonary drug delivery is an attractive route for systemic drug delivery. However, pulmonary drug delivery can be limited by poor aqueous solubility and slow drug dissolution. Insoluble particles are removed from the lungs by the mucociliary clearance in the upper airways and by macrophages in the alveoli (86). Cyclodextrins can be used in the pulmonary delivery to increase the solubility, stability and dissolution rate of water-insoluble and chemically unstable drugs. Cyclodextrins are capable to reduce the drug clearance resulting that increased drug absorption and faster onset of action. Furthermore, by forming drug/cyclodextrin inclusion complexation, a liquid drug can be converted to a solid form, bad smells and/or tastes can be reduced, two incompatible drugs can be mixed in a dry powder formulation, and local drug irritation in the lungs can be reduced. In general, cyclodextrins that are considered safe for parenteral administration are also considered safe for pulmonary administration (HP-β-CD and SBE-β-CD) (86). The number of studies dealing with pulmonary applications of cyclodextrins is also very limited. Studies have been performed using premetered dry powder inhalers, which emit the dose from a pierced blister or capsule (87). The respirable fraction of salbutamol from Diskhaler® has been increased by complexation with DM-β–CD and γ- CD (88), and the respirable fraction of beclomethasone dipropionate from Microhaler® has been increased by HP-β-CD complexation (89). Furthermore, the absorption of intratracheally administered drugs has been shown to increase in the presence of various cyclodextrins (89-91). A recent study with budesonide also showed that cyclodextrin complexes could be used in an inhalation powder

Table 5. Use of cyclodextrins and their derivatives in Transdermal and rectal drug delivery

Use of cyclodextrins and their derivatives in Transdermal route		
Cyclodextrins	**Drugs used**	**Role of cyclodextrins**
α-Cyclodextrin	Miconazole	Improvement of release and permeation
β-Cyclodextrin	Tixoxortol 17-butyrate 21-propionate Betamethasone, Norfloxacin, Indomethacin Chlorpromazine hydrochloride	Improvement of stability Improvement of release and permeation Reduction of local irritation
Dimethyl-β-cyclodextrin (DM-β-CD)	Indomethacin, Predonisolene, Sufanilic acid Chlorpromazine	Improvement of release and permeation Reduction of local irritation
Random methyl-β-cyclodextrin	Hydrocortisone, Acitretin	Improvement of release and permeation
Hydroxypropyl-β-cyclodextrin (HP-β-CD)	Dexamethasone, Miconazole, Liarozole	Improvement of release and permeation
Maltosyl-β-cyclodextrin	Hydrocortisone	Improvement permeation
Diethyl-β-cyclodextrin	Nitroglycerin	Improvement of release and permeation
Carboxymethyl-β-cyclodextrin	Hydrocortisone	Improvement of release and permeation
Carboxymethyl-ethyl-β-cyclodextrin	Prostaglandin E	Improvement of release and permeation
γ-Cyclodextrin	Betamethasone, Predonisolone	Improvement of release and permeation
Use of cyclodextrins & its derivatives in rectal drug delivery		
β-CD	Cefmetazole, Morphine hydrochloride	Improvement of release, stability and permeation
β-CD	Carmoful Naproxen, Piroxicum	Improvement of stability Improvement of release and permeation
HP-β-CD	Diazepam	Improvement of release and permeation
DM-β-CD	Carmoful, Diazepam 4-biphenylacetic acid, Ethyl 4-biphenylyl acetate	Improvement of release and permeation Reduction of local irritation
TM-β-CD	Carmoful, Diazepam	Improvement of permeation
γ-CD	Diazepam, Flurbiprofen	Improvement of release and permeation

without lowering the pulmonary deposition of the drug (92).

Parenteral Drug Delivery: Cyclodextrin derivatives such as HP-β-CD and SBE-β-CD have been widely investigated for parenteral use on account of their high aqueous solubility and are safe in parenteral administration. 40% w/v HP-β-CD containing an itraconazole parenteral injection was commercialized in the United States and Europe (93). Aqueous phenytoin parenteral formulations containing HP-β-CD exhibited reduced drug tissue irritation and precipitating tendency because their pH values were significantly closer to the physiological value 7.4 (94). Ziprasidone mesylate was developed by inclusion complexation with SBE-β-CD for IM administration with targeted concentration of 20 to 40 mg/mL (95).

Cyclodextrin applications in the design of some novel delivery systems: Cyclodextrins and their derivatives have been used in novel delivery systems such as nanoparticles, liposomes, microspheres and microcapsules.

Nanoparticles: Nanoparticles (Solid Lipid Nanoparticles and Nanostructured Lipid Carriers) are considered to be more stable than liposomal delivery systems. However, a major drawback is associated with the drug loading capacity of polymeric nanoparticles. Cyclodextrins are used in the nanoparticle development to improve water solubility and sometimes the hydrolytic or photolytic stability of drugs for better loading properties. Saquinavir-loaded nanoparticles could be easily prepared in the presence of a drug–cyclodextrin complex. It was found that large amounts of cyclodextrins remained associated with the particles, resulting in a 20-fold increase in saquinavir loading compared to nanoparticles prepared in the absence of cyclodextrins. It was shown that the loading in saquinavir of poly (alkylcyanoacrylate) nanospheres could be dramatically improved by simultaneously increasing the apparent solubility of the drug in the preparation medium and the amount of cyclodextrin associated with the particles, making these nanospheres a promising system for oral application. Thus, cyclodextrins constitute very powerful tools in drug targeting because they can increase dramatically the loading capacity of nanoparticles by improving water solubility and drug stability (96).

Liposomes: Cyclodextrin complexation can increase liposomal entrapment of lipophilic drugs. Liposomes entrap hydrophilic drugs in the aqueous phase and hydrophobic drugs in the lipid bi layers and retain drugs en route to their destination with a predetermined rate. By forming water soluble complexes, CD would allow insoluble drugs to accommodate in the aqueous phase of vesicles, thereby potentially increasing the drug-to-lipid mass ratio levels, enlarges the range of insoluble drugs amenable for encapsulation, allows drug targeting and reduce drug toxicity. Complexation with CD can also improve the stability of liposomes. Skalko, N et al., reported that nifedipine inclusion complexation with CDs increased the liposomal entrapment of nifedipine by reducing its interaction with lipid bilayers and also improved the liposomal stability in plasma (97). Stability of liposomes were improved by complexation with CDs. Skalko-Basnet, N *et al.,* (98) reported that the most stable liposomal formulations of metronidazole and verapamil were obtained by direct spray drying of lipid, drug, and HP-β-CD mixture. Inclusion complexation can greatly increase the chemical stability of labile drugs in multilamellar liposomes. Multilamellar DRV liposomes containing a riboflavin/γ-CD complex provided optimal protection to the photosensitive drug (99). Liposomal entrapment of prednisolone was higher when incorporated as HP-β-CD complex than free drug. Selection of CD have a significant effect on the amount of drug associated with vesicles, for example, HP-β-CD, with a more lipophilic interior and considerably higher aqueous solubility incorporated higher drug amounts in vesicles than β-CD. However, HP-β-CD, as a result of its ability to get entrapped in higher amounts in the vesicles, also showed a higher velocity of destabilizing effect on vesicles than β-CD.

Microspheres and Microcapsules: The role of cyclodextrins in microsphere preparation was first studied by Kang *et al.,* (100). HP-β-CD acted as a promising agent for stabilizing lysozyme and bovine serum albumin (BSA) during primary emulsification of poly (d, l-lactide-co-glycolide) (PLGA) microsphere preparation. The stabilizing effect was reported to be a result of increased hydrophilicity of the proteins caused by shielding of their hydrophobic residues by HP-β-CD; this also reduces their aggregation and denaturation by keeping them away from methylene chloride water interface. HP-β-CD enhanced BSA conformational stability and also increased its recovery from water/oil emulsion by preventing the adsorption of the protein to PLGA. Cyclodextrins were also used to modulate peptide release rate from microspheres, e.g., HP-β-CD co-encapsulation in PLGA microspheres slowed down insulin release rate (101, 102).

It was suggested that crosslinked β-CD microcapsules, because of their ability to retard the release of water-soluble drugs through semipermeable membranes, can act as release modulators to provide efficiently controlled release of drugs (94). Inclusion complexes of glycerides, fatty acids or fatty alcohols do possess surface activity and this property together with their ability to form aggregates frequently result in formation of dispersed systems (103).

Micro Scale- Interpenetrating Networks (ms-IPNs): Cross-linking of HP-β-CD with ethylene glycol diglycidyl ether (EGDE) in carbopol dispersions enabled the synthesis of cyclodextrin hydrogels with domains of interpenetrating acrylic microgels (micro scale- interpenetrating networks ms-IPNs) in a single step under mild conditions. An adequate design of the HP-β-CD /carbopol ms-IPNs provides a single material with tunable mechanical properties, in which the carbopol microgels provide the ms-IPNs with flexibility, bioadhesion, swelling ability and pH-responsiveness, while the HP-β-CD network is mainly responsible for the drug loading and the control of the release. The ms-IPNs properties can be modulated through an adequate selection of the proportion of both components, which makes them potentially useful as versatile vehicles of relatively hydrophobic substances. The stability against autoclaving also enables the sterilization of ms-IPNs (104).

Colon-Specific Drug Delivery: CDs are barely hydrolyzed and only slightly absorbed in the stomach and small intestine but are absorbed in the large intestine after fermentation into small saccharides by colonic microbial flora. The peculiar hydrolyzing property of CDs makes them useful for colon drug targeting. Biphenyl acetic acid (BPAA) prodrugs for colon-specific delivery were developed by conjugation of the drug onto one of the primary hydroxyl groups of α-, β-, and γ-CDs through an ester or amide linkage. The CD-based prodrug approach was used for delayed release and colon-specific drug delivery, e.g., the absorption of Biphenyl acetic acid from γ-CD prodrugs was found to be from caecum and colon in rats with carrageenan induced inflammation, in contrast to that from the β-CD complex, which was mainly from the small intestine. Rajeswari, C *et al.*, *in vivo* study in rats revealed that both sugar-degrading and ester-hydrolyzing enzymes are necessary for colon-specific release of butyric acid from its β-CD ester conjugates (94). Drug conjugation with α-CD resulted in a delayed release type prodrug formulation for colon-specific delivery showed the side effects of drugs while maintaining their therapeutic effect, e.g., site-specific degradation of prednisolone/α-CD conjugates in the large intestine alleviated the side effects of the drug while maintaining its anti-inflammatory action (105). Cyclodextrin based colon specific drug delivery system best suits for γ-CD prodrugs in comparison to α- and β-CD conjugates.

Brain Targetting or Brain Drug Delivery: Brewster and Loftsson (106) first discussed the use of water-soluble, chemically modified, cyclodextrin derivatives such as HP-β-CD in the formulation development of chemical delivery system (CDS). Formulation development of CDS

is based on the need for appropriate dosage form, solubility, stability, and dissolution characteristics. HP-β-CD contributed to the development and preclinical testing of several CDS by providing a stable and water-soluble dosage form suitable for parenteral administration. Use of CDs in the formulation of CDS can be demonstrated by the significantly improved solubility, stability, and pharmacologic activity of CDS of thyrotropin-releasing hormone analogs on complexation with HP-β-CD (94). The very low penetration across the BBB greatly hinders the therapeutic use of peptides, and whenever unexplainable poor peptide absorption is seen the role of the efflux pumps should be examined. Arima, H *et al.,* (107) reported that P-gp mediated peptide transport may play an important role in reducing the peptide delivery to the central nervous system *in vivo*. It was also indicated that CDs such as DM-β-CD, due to their inhibitory effect on P-gp efflux function, may enhance drug delivery to brain.

Gene Delivery: Amphiphilic and neutral as well as cationic CDs have been used for synthesis of novel gene delivery vectors. Neutral CDs like β-CD, DM-β-CD and HP-β-CDs were reported to increase DNA cellular uptake by increasing its permeability. The increased DNA permeability was reported to be a result of interaction of the CDs with membrane components such as cholesterol, but not due to their complexing ability for DNA. Cationic polyamino CDs, because of their polycationic polyanionic interaction with mononucleotides, neutralized the multiple charges on DNA and thus made DNA compact into a particle of suitable size for cellular internalization. Amphiphilic CDs, because of their vesicle-forming potential, offer an additional possibility for polar nucleotides to complex into aqueous vesicle core while allowing hydrophobic agents to complex into individual cavities or interior of the bilayer with multiple lipophilic hydrocarbon chains. Polycation polymer/DNA composite structures (Polyplexes) of linear, cationic, β-CD-containing polymers (βCDPs) were found to be suitable for DNA delivery due to their increased transfection efficiency and stability against enzymatic degradation with low *in vitro* and *in vivo* toxicity. CDs were also found to enhance plasmid or viral-vector based delivery of genes. Positively charged quaternary amino and tertiary amino β-CDs significantly enhanced the transfection efficiency of negatively charged adenoviral vector-based gene formulations. It was reported that the transfection enhancement by the cationic β-CDs could be a result of increased viral internalization caused by increased viral binding to cell and improved cell membrane permeability. CDs also enhanced the physical stability of viral vector formulations for gene therapy (108).

Cyclodextrins-Non- inclusion complexes: In the classical cyclodextrin chemistry, it has generally been assumed that the mechanism whereby cyclodextrins exert their effects, especially their augmentation of solubility, is via the formation of noncovalent, dynamic inclusion complexes. In other words, it is assumed that when a drug molecule forms a complex with cyclodextrin, then some given lipophilic moiety of the drug molecule enters into the hydrophobic cyclodextrin cavity. This is a model, which regards drug-cyclodextrin interactions as a discrete phenomenon and ignores the possible interaction of these complexes with one another. That is the hydrated drug/cyclodextrin complexes are in an ideal solution in which individual complexes are independent of each other. It is becoming increasingly apparent that such assumptions may not be universally applicable or all encompassing. Cyclodextrins are able to form both inclusion and non-inclusion complexes. In saturated aqueous solutions guest/cyclodextrin complexes frequently consist of a mixture of inclusion and non-inclusion complexes. Specifically, there is a growing body of evidence that supports the important contribution of non-inclusion-based aspects for drug solubilization by cyclodextrins including surfactant-like effects and molecular aggregation. Cyclodextrins and their complexes

form water soluble aggregates in aqueous solutions and these aggregates are able to solubilize lipophilic water insoluble drugs through non-inclusion complexation or micelle-like structures (109).

Conclusion

Cyclodextrins are useful functional excipients, which are being used in an ever-increasing way to camouflage undesirable pharmaceutical characteristics, especially poor aqueous solubility. CDs, as a result of their complexation ability and other versatile characteristics, are continuing to have different applications in different areas of drug delivery and pharmaceutical industry. However, it is necessary to find out any possible interaction between these agents and other formulation additives because the interaction can adversely affect the performance of both. It is also important to have knowledge of different factors that can influence complex formation in order to prepare economically drug/CD complexes with desirable properties. Since CDs continue to find several novel applications in drug delivery, we may expect these polymers to solve many problems associated with the delivery of different novel drugs through different delivery routes. The expanded use of known cyclodextrins as well the development of new derivatives continues to energize this field of study.

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Arsenic Toxicity and Possible Treatment Strategies: Some Recent Advancement

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Abstract

Arsenic toxicity has emerged as a global concern of prevalence especially in various Asian regions, highlighted with 130 million population at risk in India and Bangladesh. Arsenic toxicity has been associated with numerous health effects affecting almost every organ system. These adverse effects have been identified to establish or facilitate various diseased manifestations and pathological conditions that may be as severe as internal cancers. Despite the extensive research in the filed after the recognition of severity of the problem no ideal therapy is available for arsenicosis patients. Although toxicokinetic, especially the metabolism that plays crucial role in toxic advent along with molecular mechanism of arsenic toxicity is much investigated, we are far from providing clinical solution. Chelation therapy, which is recommended as the prime line of treatment in metal toxicity has been proven ineffective or inappropriate due to adverse effect limitations. Development of new drug and newer therapeutic concepts suggested by handful of research groups working in the filed provide some hope for the clinical cases of millions of arsenic poisoning patients.In the present review we have highlighted and addressed in brief arsenic-induced pathological signs and symptoms, clinical diagnosis and available therapeutic solutions and relevant hypothesis.

Keywords: Arsenic, Methylation, Oxidative Stress, hyperkeratosis, chelation therapy, DMSA, Antioxidants

Introduction

Arsenic is one of the leading cause and concern of mass poisoning in children and adults in various parts of Asia especially Bangladesh and India (1, 2). It is posted as number 1 on the Agency for Toxic Substances and Disease Registry's (ATSDR) "Top 20 List". More than 20 arsenic compounds are present in the natural environment and biological systems. Trivalent arsenic species, such as inorganic arsenite (AsIII), monomethylarsonous acid (MMAIII), and dimethylarsinous acid (DMAIII) are more toxic compared to pentavalent arsenic species (AsV) (3). In general, the toxicity of arsenic compounds is in the following order: arsine >arsenites> arsenates > organic arsenicals > elemental arsenic.Inorganic arsenic compounds, which are found throughout the environment, can cause acute and chronic toxic effects. Human may encounter arsenic in contaminated drinking water from wells drilled into arsenic rich ground strata or in water contaminated by industrial or agro chemical waste. Exposure via drinking water has been associated with cancer of the skin and various internal organs as well as hyperkertosis, pigmentation changes and effects on the circulatory and nervous system. Chronic arsenic toxicity due to drinking of arsenic contaminated water has been reported from many countries. Recently, large population in West Bengal in India and Bangladesh has reported to be affected with arsenic. The delayed health effects of exposure to arsenic, lack of common definitions and of local awareness as well as poor reporting in affected

areas are the major problems in determining the risk analysis and extent of the arsenic-in-drinking-water (4-6). Nearly 16 districts of West Bengal have been reported to have ground water arsenic concentrations above 0.05 mg/L where the WHO permissible limit amount to not more than 10ppb (7). Up to 90% of the Bangladesh population of 130 million drinks well water contained with high concentrations of arsenic. Piped water supplies are available only to a little more than 10% of the total population living in the large agglomerations and some district towns. The impact of arsenic extends from immediate health effect to extensive social and economic hardship.

Absorption, Distribution, Metabolism and Excretion: About 60-90% of soluble arsenic compounds are absorbed from the gastro intestinal (GI) tract following ingestion; inhalation exposure may be similar. Once absorbed, arsenic is stored in liver, kidneys, heart and lung while lower amount are present in muscle and neural tissues (8). In humans, absorbed inorganic pentavalent arsenic is bio-transformed to trivalent arsenic. Trivalent form of arsenic undergoes methylation to form less toxic compounds that are excreted in urine but some inorganic arsenic is excreted in the urine unchanged. Arsenic (V) is less toxic than arsenic (III). Arsenite (As III), the hydrated form of arsenic trioxide, is harmful as it is, owing to its facile covalent reaction with endogenous thiol groups especially, dithiols. Arsenic (III) is extensively bio-transformed into various methylated metabolites with markedly different toxic potential (9). Methylation of arsenic has long been regarded as a detoxification process because the pentavalent methylated arsenic metabolites; monomethylarsonic acid and dimethylarsinic acid are much less toxic and excreted more readily than As (III).

Mechanism of arsenic Toxicity: Arsenic toxicity is postulated to be primarily due to the binding of arsenic (III) to sulfhydryl group containing enzymes. Glutathione (GSH) plays a critical role in both the enzymatic and non-enzymatic reduction of pentavalent arsenicals to trivalent and in the complexation of arsenicals to form arsenicothiols during methylation process. The interaction of arsenic with glutathione and its related enzymes by changing their redox status and this may lead to the alterations of their biological function (10, 11). Recent studies have indicated that arsenic exerts toxicity by generating reactive oxygen species (ROS), but the mechanism is still unclear (12-14).

Trivalent intermediates of arsenic are involved in the formation of MMA and DMA (a pentavalent arsenic form) that might play an important role in arsenic toxicity as they are known to react with sulfhydryl groups and are highly toxic. Trivalent arsenic also inhibits pyruvate dehydrogenase (PDH), a multi sub-unit complex that needed lipoic acid as a cofactor for enzymatic activity. It has also been reported that MMA (III) is more potent inhibitor of PDH than arsenite (15). PDH oxidizes pyruvate to acetyl CoA, a precursor to intermediates of the citric acid cycle. The citric acid cycles degrade the intermediate, and this provides reducing equivalents to the electron transport system for ATP production. Inhibition of PDH may ultimately lead to decreased production of ATP. Inhibition of PDH may explain in part the depletion of carbohydrate observed in rats administered arsenic (15) (Fig. 1).

Oxidative stress is a relatively new theory of arsenic toxicity (16). Since about 1990, additional data supporting this theory and scientific acceptance of this mode of action have continued to occur. Dimethylarsine (a trivalent arsenic form) a metabolite of DMA produced by a process of reduction *in vivo* reacts with molecular oxygen forming $(CH_3)_2As^{\bullet}$ radicals and superoxide anions. Exposure to these free radicals can lead to DNA damage (single strand breaks) (17). Oxidative stress theory for arsenic carcinogenicity can also be explained by its ability to cause cancer at high rates in the lung, bladder and skin. Human lung may be an organ responsive to arsenic carcinogenesis because of high partial pressure of oxygen and the fact that dimethylarsine, a gas is excreted via the lungs. In addition, human bladder may be

another organ responsive to arsenic carcinogenesis because of high concentration of DMA and MMA that is stored in the lumen of the bladder (Fig. 1).

Signs and Symptoms of Arsenic Toxicity

Clinical Diagnosis of Arsenic Toxicity: Clinical diagnosis for arsenicosis can be done by measuring blood, urine, and hair arsenic concentration. However, these diagnostics may be sensitive to duration of exposure for example blood arsenic concentration is only reliable within few days of acute exposure. In case of chronic exposure, urinary arsenic is the best indicator of current or recent body concentrations. Hair or fingernail arsenic concentrations may be useful in evaluating past exposure. Most investigators have used hair rather than nails arsenic because the former is easier to obtain in sufficient quantities. The diagnosis of chronic arsenic poisoning must also rely on the characteristic, clinical features of the typical skin lesions, debility, weight loss and neuropathy, since hair arsenic levels are supportive of the diagnosis and not self-sufficient to obtain complete clinical picture.

It is advisable that proper investigation should be carried out to define the various manifestations in chronic arsenicosis and these include routine hematological variable like haemoglobin, total and differential count, RBC morphology, urine and stool examination, chest X-ray, electrocardiogram determination of blood sugar, urea and creatinine. The chronic arsenicosis produces protean manifestation which is evident from the report of the clinical features in 156 cases that had drinking arsenic contaminated water in West Bengal, in India. Further, although oxidative stress and other molecular biomarkers have been investigated to be used as diagnostic tools these are yet to be clinically used (18).

Systemic Effects of Arsenic Toxicity: *Haem Synthesis Pathway:* In mammalian and avian tissues the principal product of haem synthesis pathway is haemferro-protoporphyrin IX, an essential component of various biological functions including oxygen transport systems, mixed function oxidative reactions and other oxidative metabolic processes. All eight steps of the haem synthesis are catalyzed by enzymes, which require functional sufhydryl (-SH) group for optimal catalytic activity. Arsenic exposure has been known to influence the activity of several

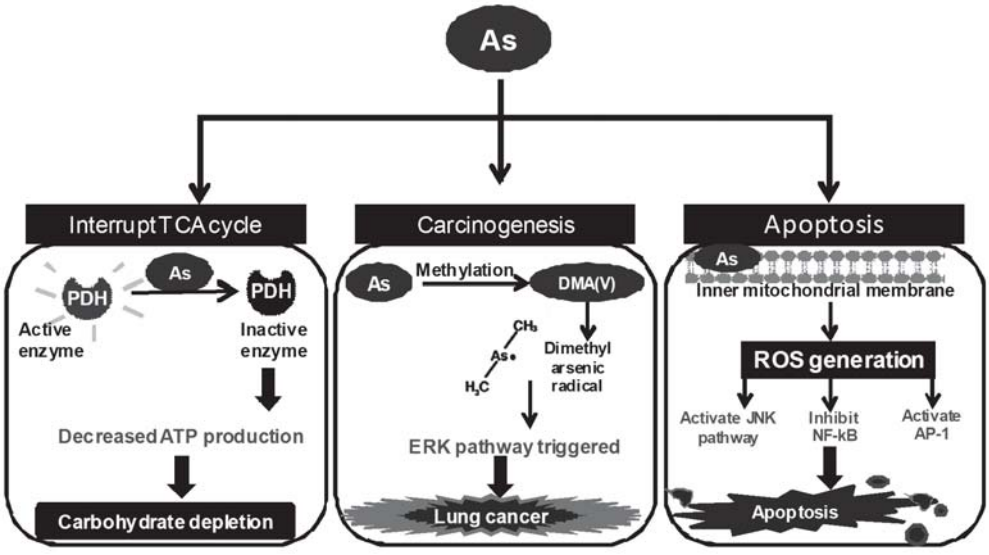

Fig. 1. Mechanism of arsenic toxicity via different pathways

enzymes of haem biosynthesis (19). It has been reported that arsenic exposure produces a decrease in ferrochelatase, and decrease in COPRO-OX and increase in hepatic 5-aminolevelinic acid synthetase activity (20). Sub-chronic exposure to arsenic has also been reported to inhibit ALA-S and ferrochelatase activities, which catalyze limiting steps in the heme synthesis pathway, leading to increases uroporphyrin (URO) and coproporphyrin and COPRO urinary excretion. Few recent studies also suggested a significant inhibition of blood d-aminolevulinic acid dehydratase (ALAD) after sub-chronic and chronic arsenic exposure (12).

Central Nervous System: In children chronic exposure to arsenic, urine level of arsenic were inversely correlated with verbal IQ scores including verbal comprehension and long term memory (21). Previous reports confirmed that arsenic could cross blood brain barrier and produces alterations in whole rat brain biogenic amines levels in animals chronically exposed to arsenite (22). Many aspects of arsenic neurotoxicity remains to be investigated from its entrance into the brain, to the cellular and molecular targets that when, altered by arsenic exposure, lead to specific central nervous system dysfunctions.

Hepatotoxicity: Arsenite is rapidly and extensively accumulated in the liver, where it inhibits NAD-linked oxidation of pyruvate or -ketoglutarate (23). This occurs by complexation of trivalent arsenic with vicinal thiols necessary for the oxidation of this substrate. Important feature of chronic arsenic toxicity in West Bengal is a form of hepatic fibrosis that causes portal hypertension, but does not progress to cirrhosis (24). Clinical examinations reveal liver to be swollen and tender. The analysis of blood revealed elevated levels of hepatic enzymes

Dermal Toxicity: Skin cancer has been associated with chronic inorganic arsenic exposure (25). Skin cancers are mostly monocenteric but sometime multicenteric cases are also found. Dermal changes most frequently reported in arsenic exposed humans include hyper pigmentation, melanosis, hyperkeratosis, warts, and skin cancer.

Carcinogenic Effects: One of the most severe adverse manifestations of chronic arsenic poisoning appears to be cancer (26). Numbers of epidemiological studies have reported a strong correlation between environmental, occupational and medical exposure of man to inorganic arsenic and cancer of skin and lungs. Epidemiological studies too have shown that chronic exposure to arsenic can result in an increased incidence of cancer of the lung, skin, bladder and liver (27-29). Arsenic-induced cancer has been extensively investigated and oxidative stress appears to be one of the most convincing mechanism underlying the etiology and progression of disease (30).

Developmental and reproductive toxicity: Chronic studies did not report any male reproductive organ pathology. However, in human studies a correlation has been observed between arsenic exposure and incidence of abortion. Higher spontaneous abortions and stillbirths were reported in the high arsenic area (arsenic in drinking water > 0.1 mg/L) compared to the control areas (31, 32).

Therapy

Chelation Therapy: Chelation is the formation of a metal ion complex in which the metal ion is associated with a charged or uncharged electron donor referred to as ligand. Chelators act according to a general principle: the chelator form a complex with the respective (toxic) ion and these complexes reveal a lower toxicity and more easily eliminated from the body.

2, 3-dimercaprol (BAL) is a traditional chelating agent that has been used clinically in arsenic poisoning since 1949 (33). Beside rapid mobilization of arsenic from the body, it causes a significant increase in brain arsenic. Other side effects include vomiting, headache, lachrymation, rhinorrhea and salivation, profuse sweating, intense pain in the chest and abdomen and anxiety. One of the chemical derivatives of

dimercaprol (BAL) is DMSA. DMSA is an orally active chelating agent, much less toxic than BAL and its therapeutic index is about 30 times higher. No significant loss of essential metals like zinc, iron, calcium or magnesium has been reported with DMSA administration. However in a double blind, randomized controlled trial study conducted on few selected patients from arsenic affected West Bengal (India) regions with oral administration of DMSA suggested that DMSA was not effective in producing any clinical and biochemical benefits or any histopathological improvements of skin lesions (34). Its distribution is predominantly extracellular; ultimately it is very well able to chelate arsenic from extracellular sites but does not able to chelate arsenic from intracellular sites. Thus, in lack of an arsenic chelator, researchers have been suggesting alterative therapeutic solutions for effective arsenic removal from body and clinical recovery.

Combination Therapy: A new trend in chelation therapy has emerged recently, which is to use of combination therapy with more than one chelating agent instead of monotherapy (35-37). Vitamins, essential metals or amino acid supplementation during chelation therapy has also been found beneficial in increasing metal mobilization and providing recoveries in number of altered biochemical variables. Combined treatment with a chelating agent having antioxidant property and a thiol chelator could be a better treatment protocol for arsenic poisoning compared to monotherapy with a chelator (38, 39). Flora and his group shave worked extensively in the field of arsenic therapy. They reported that co-administration of naturally occurring vitamins like vitamin E or vitamin C during administration of a thiol chelator like DMSA or MiADMSA may be more beneficial in the restoration of altered biochemical variables (particularly the effects on haem biosynthesis and oxidative injury) although it has only limited role in depleting arsenic burden. It was observed that optimum effects of chelation therapy could be achieved by combined administration of DMSA and MiADMSA (38, 40). It is evident from above that combination therapy is a new and a better approach to treat cases of metal poisoning (Fig. 2).

In search of an effective arsenic chelating agent, some mono and diesters of DMSA have been developed and tried against cases of

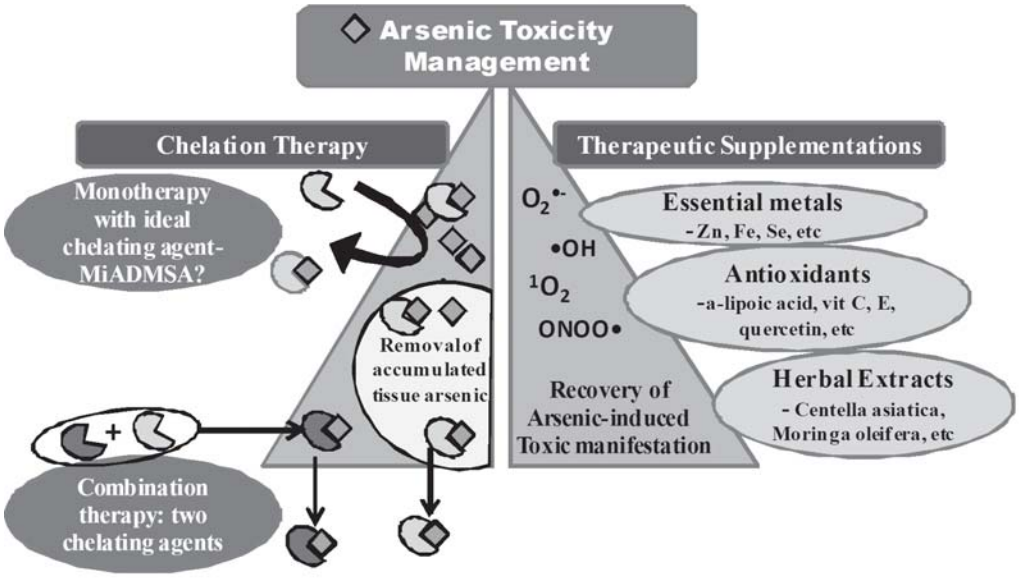

Fig. 2. Various therapeutic strategies in use against arsenic toxicity

experimental heavy metal poisoning. Monoisoamyl ester of DMSA (MiADMSA), a C_5 branched chain alkyl monoester of DMSA has found to be the most effective (41) against chronic arsenic toxicity. The metal chelators are given to increase the excretion of arsenic but unfortunately use of these chelators is comprised by number of drawbacks (42). These drawbacks open the search for new treatment which has no side effects and maximum clinical recovery in terms of altered biochemical variables because the total elimination of metals from the environment is not feasible.

Therapeutic supplementation: Chelation therapy forms mainstay for arsenic toxicity which must be developed and recommended as first line of therapy. However, for complete clinical recoveries various therapeutic supplementations also known as adjuvant have been suggested.

Nutritional intervention: Experimentally, excesses or deficiencies of essential trace elements and other dietary nutrients facilitate arsenic absorption. Selenium can also alleviate arsenic toxicity. Selenate partially prevents the uncoupling of oxidative phosphorylation by arsenate and decreases the teratogenic toxicity of arsenate in hamsters when both salts were injected simultaneously. Arsenic can also induce metallothionein (MT), a low molecular weight cysteine rich metal binding protein. This implies that arsenite can be detoxified by MT (43). Dietary antioxidants such as vitamin E and vitamin A may also be alleviating arsenic toxicity. Addition of vitamin E could atleast in part prevent the arsenic-induced sever health manifestations (Fig. 2).

Role of Antioxidants: Oxidative stress has been popularly associated with metal toxicities and relevant diseased manifestations (44). Thus, employing antioxidants as therapeutic supplementations during metal chelation therapy serves beneficial effects. Antioxidants are substances, which inhibit or delay oxidation of a substrate while present in minute amounts. Nutritional antioxidants act through different mechanisms and in different compartments, but are mainly free radical scavengers: They directly neutralize free radicals, reduce the peroxide concentrations and repair oxidized membranes (39). They quench iron to decrease ROS production, via lipid metabolism, short-chain free fatty acids and cholesteryl esters neutralize ROS (45). Typical natural antioxidants include tocopherol, ascorbic acid, flavonoides, quercetin, carotene, cinnamic acid, peptides and phenolic compounds. Quercetin has been shown to scavenge superoxide radicals, protect from lipid peroxidation and chelate metal ions to form inert complexes (46).

Role of herbal Products: Plants parts like wood, bark, stem, leaf and pod may be important source of natural antioxidants. *Aloe vera* has been reported to possess antiulcer, antidiabetic, antioxidant and free radical scavenging activity (47). *Centella asiatica* improves learning and memory and possess antioxidant, antiulcer, and radioprotective activity. Thus, these herbal extracts when evaluated showed protection against arsenic-induced said manifestations (48-54). It is proven recently that shelled *Moringa oleifera* seed powder has ability to remove cadmium and arsenic from the aqueous system. Fourier transform infrared (FTIR) spectrometery highlights protein/amino acid – arsenic interactions responsible for sorption phenomenon of seed powder of *Moringa oleifera* (55-58). Garlic has been reported to prevent arsenic-induced oxidative stress and apoptosis and reversing altered clinical variables (59-61).

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Effect of Concentration and Ionic Strength on Pathway of Bovine Insulin Fibril Formation

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Abstract

The effect of experimental conditions on the formation of structural intermediates and pathway of bovine insulin fibril formation was studied at pH 0.5 and 60°C. The relative amount of oligomeric intermediates observed during fibril formation was found to depend on both ionic strength and protein concentration. At a protein concentration of 1mg/mL, significant amounts of oligomeric intermediates including spherical assemblies and protofibrils were detected by AFM under the condition of 0.35 ionic strength. The oligomeric intermediates dissociated upon cooling for 10 days at 25°C. At 0.15 ionic strength, fibril formation proceeded without generation of oligomeric intermediates. Amorphous aggregates were observed prior to detection of well-defined fibrils under this condition. Increasing protein concentration from 1 to 2 mg/mL at 0.15 ionic strength or decreasing the protein concentration from 1 to 0.5 mg/mL at 0.35 ionic strength resulted in generation of similar low amounts of oligomeric intermediates. Fibril formation at 0.35 ionic strength and 1mg/mL of insulin was consistent with a nucleated conformational conversion mechanism, while fibril formation at 0.15 ionic strength and at the same protein concentration followed a nucleated polymerization mechanism. The results from this study show that ionic strength and protein concentration determined the relative quantities of structural intermediates formed during the early stages of fibril formation and the pathway of fibril assembly.

Keywords: Bovine insulin, fibril, protofibril, AFM

Abbreviations: ThT, thioflavin T; AFM, atomic force microscopy; TEM, transmission electron microscopy; DLS, dynamic light scattering; NP, nucleated polymerization; NCC, nucleated conformational conversion.

Introduction

Heating insulin in acidic solution or exposing to nonpolar surfaces such as air or plastic tubings predisposes the protein to rapidly form fibrils *in vitro* (1-5). Insulin fibrils are found to be unbranched, curved or linear, 3-4 nm in diameter with lengths reaching up to several microns (6-9). Dense crystal packing of the exposed hydrophobic surfaces between insulin molecules (*7*) results in the consistent size and compact shape of the basic structure of the insulin fibril called the protofilament (8). Several protofilaments can further aggregate laterally into bundles or twist into braids (6, 10), depending on the type of acidic media (i.e. HCl, H_2SO_4) and the process of fibril formation either by heat or agitation (11). Under certain experimental conditions, insulin can arrange spherically into formations called spherulites, which are composed of radially oriented fibrils around a core of less structured molecules (12, 13). Circular or ring shaped insulin fibrils have also

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been observed after exposure to high pressure (14).

A simple model of insulin fibril formation has been proposed, which includes generation of a stable nucleus followed by growth of the nucleus into fibrils (12, 15). Growth or extension of the fibril is consistent with a first-order process (16) and is believed to propagate through a series of interactions between insulin molecules (8). Nuclei formation is hypothesized to be caused by the structural intermediates (7, 11, 16). These structural intermediates have been detected and characterized by NMR (17) and FTIR (10) and shown to retain much of the native insulin alpha-helical conformation. Recently, AFM was used to obtain detailed images of a unique insulin fibril assembly pathway in which short and thick seed-like forms appeared to function as lateral scaffolds for fibril growth (18).

Although significant progress has been made towards unraveling the mechanism of insulin fibrillation, much remains to be learned about the intermediates of the process and their role in protein fibril transformation (15). This study focuses on the relationship between experimental conditions (protein concentration, ionic strength), oligomeric intermediate formation, and fibril assembly pathway to further understand the mechanism of insulin fibril formation.

Experimental Procedures

***Protein and Reagents*:** Bovine insulin, glutaraldehyde, NaCl, uranyl acetate, and thioflavin T (ThT) were purchased from Sigma-Aldrich (St Louis, MO). Bovine insulin was used without further purification. Reagent grade HCl was obtained from VWR International (Brisbane, CA). Mica disks and Formvar grids were purchased from Ted Pella, Inc. (Redding, CA).

***Preparation of Samples*:** Stock solutions (0.5, 1 or 2 mg/mL) were prepared by dissolving bovine insulin into freshly-prepared 0.12 mM aqueous solutions of HCl (pH 0.5 at 60°C). The ionic strength was adjusted to either 0.15 or 0.35 using NaCl. Protein concentrations were determined by UV (276 nm) using an extinction coefficient of 1.0 for 1 mg/mL insulin (19). Aliquots of 0.5 mL protein solution were dispensed into 0.6 mL polypropylene centrifuge vials, sealed with Teflon tape, and incubated in 60ºC water bath to initiate fibril formation. At different time points, samples were removed from the water bath and analyzed by DLS, AFM, TEM, and ThT fluorescence at room temperature. Oligomeric intermediates generated in samples heated from 0- 60 minutes at 1 mg/mL and 0.35 ionic strength were then incubated for 10 days at 25°C and reanalyzed by DLS and ThT fluorescence spectroscopy.

Atomic Force Microscopy (AFM): Tapping mode AFM was conducted using a Nanoscope 3a Multimode system (Digital Instruments/Veeco, Santa Barbara, CA). Protein suspension (10µL) was deposited onto freshly cleaved mica and allowed to adsorb for one minute. The mica was rinsed with 200 µL of deionized water and dried with nitrogen gas. Tapping mode scans were taken in air, under ambient conditions at a frequency of 2.44 Hz using TESP tips (Digital Instruments). Images were processed and rendered using the Digital Instruments Nanoscope software. Images displayed as surface plots were contrast enhanced, low-pass frequency filtered and digitally zoomed from full size (600 nm) images.

Transmission Electron Microscopy (TEM): Electron micrographs were taken on Tecnai 12 transmission electron microscope (FEI, Hillsboro, Oregon) at 100-200 nm resolution. A 20 µL volume of sample was deposited onto Formvar coated grids and allowed to set for one minute before an equal volume of 0.5% (v/v) glutaraldehyde solution was added for an additional one minute. The grid was then rinsed with 4-5 drops of water and wicked dry with tissue before staining with 10 µL of 2% (w/v) of uranyl acetate for two minutes and finally wicked dry again with tissue.

Dynamic Light Scattering (DLS): DLS measurements were performed using a Dynapro 99 apparatus (Proterion Corp., Piscataway, NJ). Analyses of particle size distribution of insulin

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were determined using the solid sphere model and regularization method of the Dynamic V6 software. Protein samples were analyzed without filtration.

Thioflavin T (ThT) Fluorescence Spectroscopy: ThT fluorescence measurements were conducted using a Photon Technology International fluorimeter (Lawrenceville, NJ). Aliquots of 200 mL of thoroughly mixed sample suspensions were diluted into 2.5 mL of ThT (20 mM) phosphate buffer (pH 6.0). The samples were excited at 440 nm and fluorescence intensities at emission wavelength 480 nm were recorded.

Results

Fibril Formation at High Ionic Strength: Oligomeric intermediates (some in the shape of protofibrils) were produced in solution conditions of 1 mg/mL, 0.35 ionic strength, pH 0.5, and 60°C and then examined by AFM. Fig.1, shows a progression of structures formed during insulin fibrillation. The AFM image at time 0 shows a uniform layer of nonaggregated protein molecules. At 20 minutes, variable-sized spherical assemblies either attached or situated in proximity with one another are detected. A surface plot of the 20 minute sample (labeled 20 min*) showed structures that were similar to those designated as nucleation units found in the fibril formation of other amyloid proteins (20-23). The 60 minute sample contained short string-like assemblies. The surface plot of the 60 minute sample (labeled 60 min*) revealed various structures, including protofibrils and oblong globular forms fused with neighboring protein aggregates. By 110 minutes, a net of highly branched immature fibrils were evident. The 255 minute sample showed long, thin, unbranched fibrils emerging from the more slender but highly thickened mesh of fibrous insulin.

DLS was used to monitor the growth of oligomeric intermediates in solution. Prior to heat treatment (0 minute), light scattering showed a population with mean particle size 2.2 nm (Fig. 2). The small population peak at 0.1 nm was sometimes observed in pure water and so

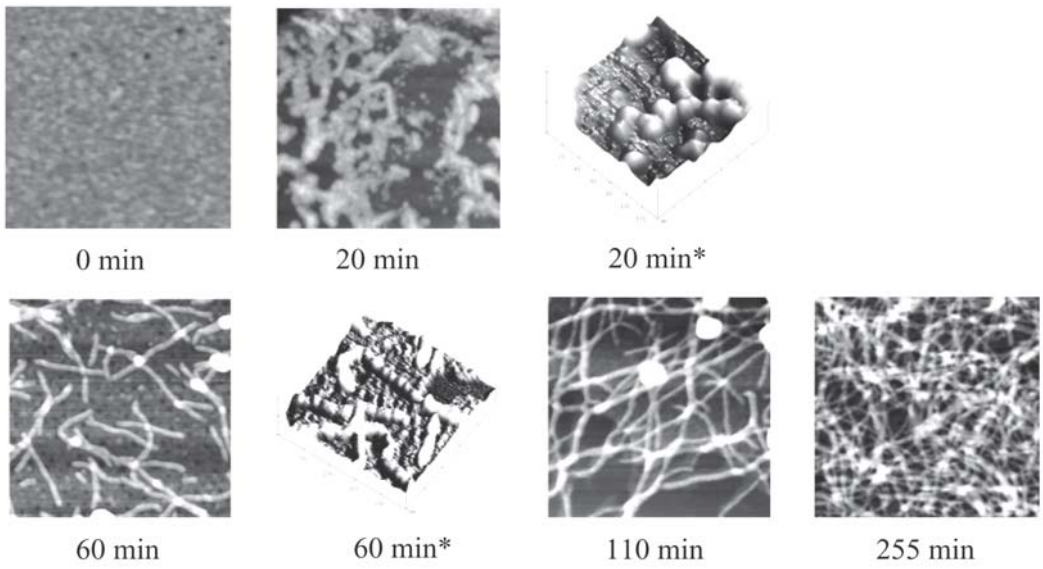

Fig. 1. Temporal evolution of oligomeric intermediates and fibrils illustrated by AFM images (600 x 600 nm). Fibrils were generated by heating insulin (1 mg/mL, ionic strength 0.35, pH 0.5) at 60°C for the indicated times. Images labeled with asterisk symbol (*) are enlarged and contrast enhanced surface plots of species detected at the indicated times.

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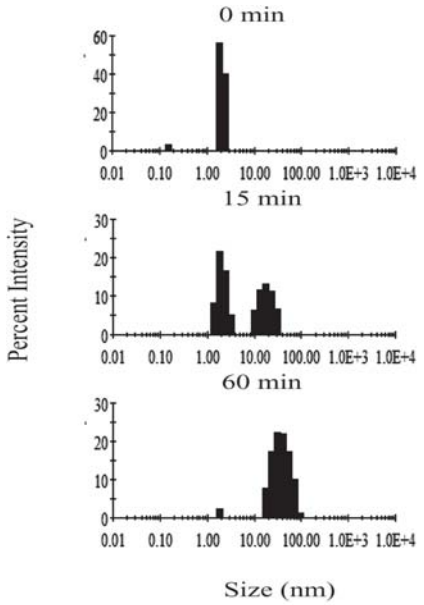

Fig. 2. Histograms of particle size distribution of samples heated at 60°C for the indicated times. Solution conditions: 1 mg/mL, ionic strength 0.35, and pH 0.5.

discarded. After 15 minutes of incubation, a second population with a mean size distribution of 14.4 nm was evident. This second population correlates temporally with the spherical assemblies observed by AFM in the 20 minutes sample (Fig.1). At 60 minutes, the second population increased in dimension and scattering intensity to reach an average size of 37 nm. Large micron sized particles also appeared as a third population in these samples.

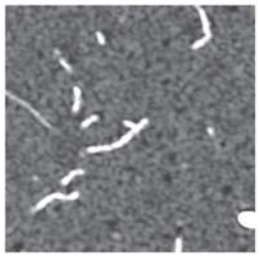

Fig. 3. AFM image (600 x 600 nm) of protofibrils assembled under solution conditions of 0.5 mg/mL insulin, ionic strength 0.35, and pH 0.5 incubated at 60°C for 60 minutes.

When the insulin concentration was reduced by half to 0.5 mg/mL while maintaining the ionic strength, pH, and temperature at 0.35, 0.5, and 60°C, protofibril-shaped oligomeric intermediates were observed by AFM. However, the amount of oligomeric intermediates generated at 60 minutes was significantly less than that generated from the 1 mg/mL sample incubated for the same length of time (compare Fig. 3 to the 60 min sample in Fig.1). Even accounting for the decrease in protein concentration there was less than half the number of protofibrils present in a typical scanned image of the same size.

Fibril Formation at Low Ionic Strength: Reducing the solution ionic strength to 0.15 at 1 mg/mL, pH 0.5 and 60°C resulted in the generation of structures different than the oligomeric intermediates detected at higher ionic strength after the 60 minutes lag time. Fig. 4B shows an AFM image of the 60 minute sample.

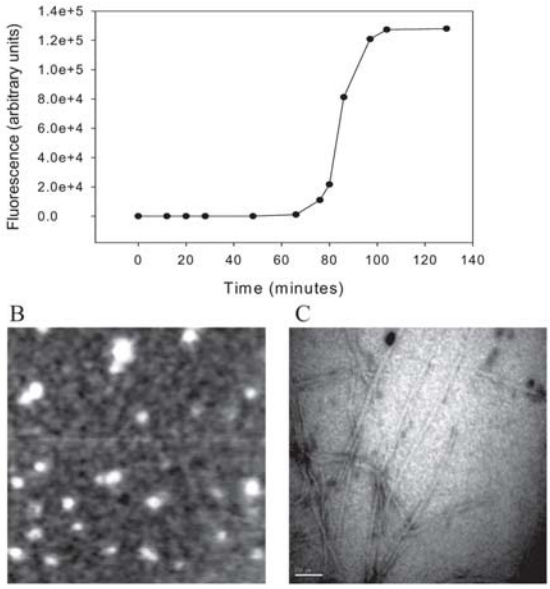

Fig. 4. ThT fluorescence profile of insulin fibrillation for solution condition of 1 mg/mL insulin, ionic strength 0.15, pH 0.5, and 60°C (A). AFM (600 x 600 nm) image of amorphous aggregates detected at the 60 minutes lag time (B). TEM of fibrils generated at 150 minutes (C). Scale bar is 100 nm.

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As shown, nucleation units and protofibrils were not detected under these conditions. Instead, only variable sized amorphous aggregates are observed. The kinetics of insulin fibril formation as determined by ThT fluorescence showed an 80 minute lag time (Fig. 4A) for the formation of fibrils. Rapid fibril growth occurred over the following 20 minutes and reached a plateau by 100 minutes of incubation. TEM was used to confirm the presence of mature fibrils at the end of a 2.5 hour incubation period (Fig. 4C).

Increasing the protein concentration from 1.0 to 2.0 mg/mL resulted in the generation of protofibrillar oligomeric intermediates at 0.15 ionic strength, pH 0.5, and 60°C. Figures 5A and B shows AFM images of insulin protofibrils observed for samples incubated for 60 minutes. TEM confirmed the presence of abundant fibrils at the end of a 2 hour incubation period (Fig. 5C).

Oligomeric Intermediate Causes Increase in ThT Fluorescence: Oligomeric intermediates generated during the early stages of fibril formation caused increased ThT fluorescence. The elevated ThT fluorescence of these samples returned to base line levels upon cooling to 25°C. ThT fluorescence was measured for samples which were shown to possess significant amounts of oligomeric intermediates as determined by AFM. Samples at 1 mg/mL of insulin, 0.35 ionic strength, and pH 0.5 were heated for 35 and 45 minutes and analyzed by ThT fluorescence. The black bars in Fig. 6 show a 10 and 75-fold increase in fluorescence intensity over baseline (value at time 0) determined for the 35 and 45 minute heated sample, respectively. The gray bars represent the fluorescence intensities remaining in the same samples after cooling for 10 days at 25°C. All samples exhibited near baseline fluorescence intensity values after cooling. No apparent visible precipitates were observed in the cooled samples, and DLS showed disappearance of the oligomeric intermediate size species from solution (Fig. 7). As expected, samples that were never heated (time 0) and stored for 10 days at 25°C showed only the insulin dimer population. Samples heated for 35 minutes and then stored for 10 days at 25°C showed dimeric insulin and micron sized particles. The samples heated for 45 minute and analyzed after storage showed only the dimeric insulin population.

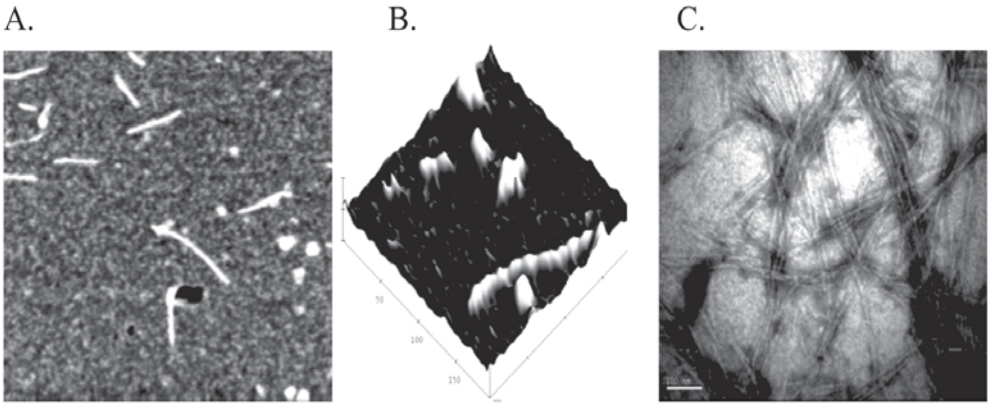

Fig. 5. Protofibrils and fibrils generated under solution condition 2 mg/mL, ionic strength 0.15, pH 0.5 and 60°C. (A) AFM image (600 x 600 nm) and (B) surface plot (200 x 200 nm) showing protofibrils present in samples heated for 60 minutes. (C) TEM image of fibrils generated after 2 hours of incubation.

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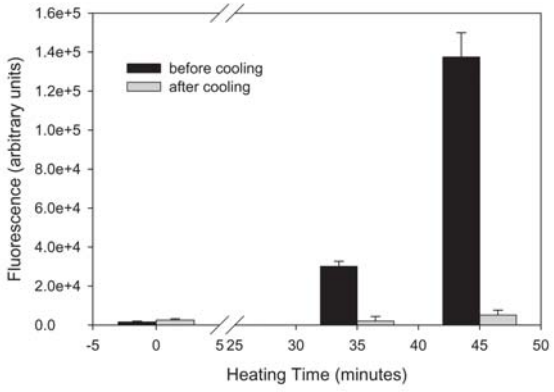

Fig. 6. Thioflavin T fluorescence detected after heating insulin at 60°C for the times indicated on x-axis (black bars). Gray bars show same analysis of the samples after further incubation at 25°C for 10 days. Solution conditions: 1 mg/mL, ionic strength 0.35, pH 0.5.

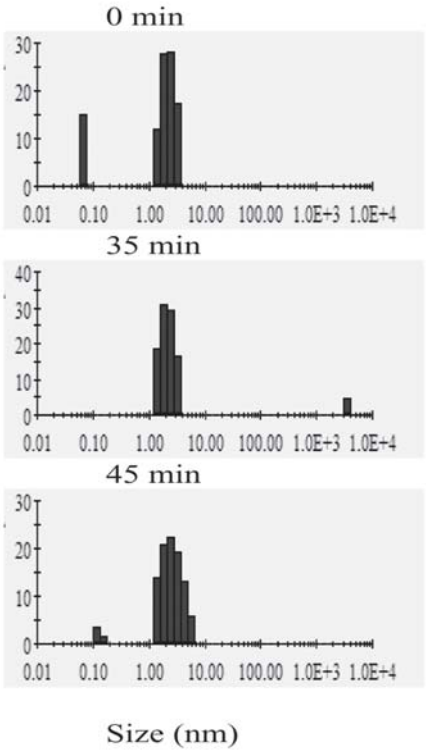

Fig. 7. Histograms of particle size distribution of samples heated at 60°C for the indicated times and then after further incubation at 25°C for 10 days. Solution conditions: 1 mg/mL, ionic strength 0.35, pH 0.5.

Discussion

Insulin remains a classic model protein for examining the fibril forming process under conditions of elevated temperature and acidic pH. The mechanism of fibril formation is primarily studied for strategies to prevent or reverse the process. Structural intermediates are investigated as possible targets for inhibition of fibril formation. Although intermediates have been found under a variety of conditions (10, 11, 16-18, 24-26) during the insulin fibril formation, its role in fibril formation remains controversial. This study was designed to explore the effect of solution ionic strength and protein concentration on the formation of structural intermediates during the early stages of insulin fibril formation and its significance in insulin fibrillation at elevated temperature and acidic pH.

Results from this study show that solution ionic strength played an important role in determining the type of structures generated during bovine insulin fibril formation. AFM revealed formation of oligomeric intermediates in shapes of spherical assemblies and protofibrils produced by conditions of elevated ionic strength as shown in Fig. 1 (solution condition: ionic strength 0.35, protein concentration 1 mg/mL, pH 0.5, and incubation temperature 60°C). These intermediates are visually similar to those reported for other amyloid proteins (20, 27).

Time sequence analysis of insulin aggregate formation by DLS and AFM provided insight to the pathway of insulin fibril formation and the role of the intermediates. Evidence from this study suggests that the oligomeric intermediates are on-pathway to fibril assembly. DLS detected a single 2.2 nm population consistent with the dimeric form of insulin in solution (16) for the unheated sample at time 0 (Fig. 2). Oligomeric intermediates were detected at 15 minutes as a single population averaging 14.4 nm giving a similar scattering intensity to that of the nonaggregated dimeric insulin population. Intermediates in the form of spherical assemblies were observed by AFM at 20 minutes (Fig. 1). These intermediates grew in size with

an average diameter of 37 nm and increased in relative number as determined by the greater scattering intensity of the oligomeric intermediates population compared to insulin dimers after 60 minutes of incubation. Abundant intermediates in the form of beaded protofibrils and elongated globular shapes were visualized by AFM after 60 minutes (Fig.1). We believe that the elongated globular structures are protofibrils that have attained increased bonding between neighboring spherical assemblies. The fibrous net-like material observed at 110 minute suggests that maturation and growth may occur partially through association of the shorter elongated globular forms. Realignment of protein molecules within the globular structure can explain the more slender appearance of the matured netted material. Maximal reorganization of the insulin molecules would result in the finer, more compact size of the fibril, as observed in the emerging fibrous mesh at 255 minutes (Fig. 1). Bovine insulin fibril assembly under this experimental condition is consistent with the nucleated conformational conversion mechanism proposed for other amyloid proteins (20, 28, 29). Oligomeric intermediates in the shape of spherical assemblies for bovine insulin were also observed recently at the very early stages of fibril formation by Jansen *et al.* (18). Under the experimental conditions used by Jansen, the pathway of fibril assembly included the generation of short and thick seed-like structures providing lateral scaffolds for fibril growth (solution condition: 1 mg/mL, pH 1.6, 60°C).

In this study, the reversible characteristic of oligomeric intermediates was demonstrated using ThT fluorescence analysis, and supported by the size analysis using DSL and by visualization using AFM. Fig. 6 compares the ThT fluorescence intensities caused by oligomeric intermediates generated after heating samples up to 45 minutes at 60°C (black bars) with the samples analyzed after further incubation at 25°C for 10 days (gray bars). The fluorescence intensities detected in the samples after cooling returned to baseline values, while the oligomeric intermediate sized population disappeared as determined by DLS (Fig. 7). The dominant scattering intensity in all the cooled samples belonged to insulin dimers. The micron sized peak observed in the 35 minute sample can be attributed to amorphous aggregates that were formed or fibrils that were nucleated during the 10 day incubation period at 25°C. These results show that insulin oligomeric intermediates are distinguished from properly folded native insulin by their capacity to induce ThT fluorescence and from fibrils by their ability to reversibly dissociate. Induction of ThT fluorescence has also been reported for Alzheimer's Ab (1-40) protein intermediates (30). Unlike fully assembled insulin fibrils, which do not readily dissociate (15) or decrease in ThT fluorescence intensity (31), insulin oligomeric intermediates were noted to reversibly dissociate with a consequent reduction in ThT fluorescence upon cooling to ambient temperature.

Under experimental conditions with lower ionic strength (solution condition: 0.15 ionic strength, 1 mg/mL, pH 0.5 and 60°C), bovine insulin fibril formation proceeded without generation of oligomeric intermediates. Examination of the species detected by AFM from the 60 minute sample revealed only amorphous aggregates (Fig.4B). The amorphous aggregates were not observed to increase ThT fluorescence as noted by the baseline value plotted in the fluorescence profile in Figure 4A. After a lag time of 70 minutes, the fluorescence intensity rapidly increased and reached a plateau at 100 minutes. This material consisted of long straight fibrils as confirmed by TEM (Fig. 4C). The sigmoid shape of the fluorescence curve shows classic fibril forming kinetics and is commonly associated with a nucleated polymerization mechanistic model of fibril assembly, in which rapid fibril growth occurs after successful formation of the nucleus (27). Bouchard and coworkers described a similar mechanism of bovine insulin fibril formation under a different set of experimental conditions (2 mM insulin, pD 2.67, 70°C) (10). Using TEM, they

detected clusters of amorphous aggregate at the early stages of fibril formation, which later disappeared with the concurrent increase in the density of fibrils. Although fibrils emerging from the amorphous clusters can sometimes be observed, no strong evidence supports the amorphous clusters to be intermediates on-pathway to fibril assembly.

To further understand the effects of protein concentration and ionic strength on fibril formation, we examined samples with various initial insulin concentrations and ionic strengths. Increasing the protein concentration from 1 to 2 mg/mL at lower solution ionic strength condition (0.15 ionic strength, pH 0.5, 60°C) resulted in production of a small amount of protofibrillar oligomeric intermediates detected in the 60 minutes sample (Fig. 5A and B). For this sample, TEM showed abundant fibrils after 2 hours of incubation (Fig. 5C). Decreasing the protein concentration from 1 to 0.5 mg/mL but at higher ionic solution strength (0.35 ionic strength, pH 0.5, 60C) also generated similar amounts of oligomeric intermediates in the 60 minute sample (Fig. 2). These results show that the relative amount of oligomeric intermediates generated under the different experimental conditions is a function of both solution ionic strength and protein concentration. Higher protein concentration and higher ionic strength are more conducive to oligomeric intermediate formation. The effect of ionic strength on oligomeric intermediate formation can possibly be explained by a molten globule conformation of proteins in the presence of high salt concentrations (32-35). The propensity for molten globules to aggregate increasing the chances for oligomeric intermediates to form would explain the dependence on insulin protein concentration.

This study demonstrates the importance of environmental conditions on the structural intermediates that are formed during bovine insulin fibril formation. The different pathways of insulin fibril assembly observed in this study and those previously reported in other studies highly suggest a multi-pathway mechanism of insulin fibril formation that is dependent on solution conditions.

Conclusion

Ionic strength and protein concentration significantly affected the types of structures that were formed during the early stages of fibril formation, and subsequently determined the pathway of fibril assembly.

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Amazonian Biodiversity: Pigments from *Aspergillus* and *Penicillium*-Characterizations, Antibacterial Activities and their Toxicities

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Abstract

The *Aspergillus* and *Penicillium* culture collection were screened for pigment production. The antimicrobial activity was measured by the agar diffusion methodology. The organic extracts (hexane, ethyl acetate and ethanol 95%) were tested for bioautography against yeasts and pathogenic bacteria. The extracts were also tested on *Artemia salina* toxicity. *Cryptococcus laurentii* and *Mycobacterium smegmatis* exhibited higher sensitivity to the pigments as test-microorganism. Pigments from *Penicillium simplicissimum* DPUA 1379 and *Penicillium janczewskii* DPUA 304 showed the highest degree of mortality for *Artemia salina* larvae. The other tested fungal strains of *Aspergillus* and *Penicillium* producing pigments, isolated from Amazonia, showed significant antimicrobial activities and total absence of toxicity.

Keywords: Colorants, Pigments, *Aspergillus, Penicillium,* Antagonism, Toxicity.

Introduction

In pigment industrialization (1,2), at present, there are many impediments such as the increasing cost of the raw materials and the energy sources for production that interfere economically in the process, besides the damage to the environment caused by effluent generation (3-9). Many fungal pigments were isolated in different environment exhibiting interesting medical applications (6-8, 10-21). An endophytic fungal pigment screening against human pathogenic bacteria was optimized to improve growth and antimicrobial pigment production. One of the most efficient isolated strains was identified as *Monodictys castaneae*. The best medium was Czapek yeast extract agar/Czapek yeast extract broth (CYA/CYB). Antimicrobial activity of the *M. castaneae* pigment significantly inhibited the growth of human pathogenic bacteria viz. *Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi* and *Vibrio cholerae*. The pigment was more active than streptomycin (22). Fungi isolated from fruits (23) and from fresh water (24) were able to produce pigments with antibacterial activities.

In this context, interests are addressed for the natural pigment sources due to the reduction of adverse effects to health and the largest consumer acceptability, when compared to the artificial pigments used in the food and cosmetics and by the pharmaceutical industries (25,26). Alternatives for production of natural pigments are microorganisms such as bacteria, filamentous fungi and yeasts. Among the filamentous fungi, *Aspergillus* and *Penicillium* are common in soil and foods, and are frequently mentioned in ecological studies (27,28). However, these fungi can cause pathologies such as breathing allergies, due to inhalation of the spores, and mycotoxicosis due to mycotoxin

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ingestion. Certain species have been the subject of much research because their potential application as sources of enzymes and antibiotics, among other natural products of industrial importance (28-34).

Thus the aim of this work was the screening of *Aspergillus and Penicillium* fungal species which produce non-toxic bioactive pigments with the best potential for possible industrial application.

Material and Methods

Microorganisms: To select pigment fungi producers, 30 cultures of *Aspergillus* and 30 of *Penicillium* were donated by the Cultures DPUA Collection of the Parasitology Department of the Federal University of Amazonas–UFAM (35) (Fig. 1). The species authentication was accomplished based on in the morphologic characteristics suggested by Raper and Thom (36), Raper and Fennel (37), Pitt (38), Samson et al. (39), Klick and Pitt (40) and Samson et al. (41).

Pigment production in solid medium: To identify the species of pigment producers, *Aspergillus* and *Penicillium* were cultivated in CYA medium and yeast extract sucrose agar (YES) medium, in Petri plates (90 mm x 10 mm), as described by Raper and Fennell (37) and Pitt (38). The cultures were maintained at 25°C for seven days and a positive result was determined by observation of pigment in the culture medium.

Pigment production in liquid medium in a bioreactor: *Penicillium melinii* DPUA-1391 cultured on YES liquid medium (20 g of yeast extract and 150 g of sucrose in 1000 mL of distilled water). The fungus was inoculated into a 1.5 L bioreactor (Bioflow-New-Brunswick) and grown with stirring at 25 °C for 7 days following a modified methods from as previously describe by Ariza et al. (42).

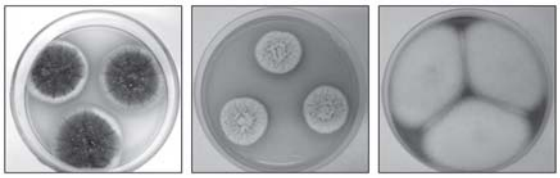

Fig. 1. Pigment production in CYA medium and YES medium for *Aspergillus* and *Penicillium* species, storage in Cultures DPUA'S Collection

Pigment extraction: After *Aspergillus* and *Penicillium* culture, 5x5 mm disks were withdrawn from the central area of the culture and were submitted to successive pigment extraction by hexane, ethyl acetate and 95% (v/v) ethanol for 48 h the solvents were evaporated at low pressure.

Preliminary chemical characterization of the most active and least toxic pigments: The extracted pigments were analyzed by liquid chromatography-mass spectrometry (LC-MS/MS, Waters UPLC Acquity - TQD Quattro Micro API).

Antimicrobial activity determination in solid media: *Aspergillus* and *Penicillium* cultures obtained in YES medium, 25°C/7 days, were analyzed against five test microorganisms [(*Candida albicans* DPUA 1336 and *Cryptococcus laurentii* DPUA 1501 cultivated in Sabouraud-agar medium, at 25°C/48 h) and (*Staphylococcus aureus* CCT 1352, *Escherichia coli* CCT 0547 and *Mycobacterium smegmatis* PDUFPE-71 cultivated in Müeller-Hinton agar medium, at 37°C/24 hours)]. From each test-microorganism an aliquot was removed to obtain a cellular suspension similar to concentration N°. 1 of MacFarland's scale. As standards, itraconazol and rifampicina (5 ug/mL) were used. For determination of the antimicrobial activity by the Block Gelose method for agar diffusion the Teixeira (35) methodology was followed. The antibacterial activity was determined by measuring the diameter of the halo around the culture disks.

Minimum Inhibitory Concentration (MIC): The microorganisms active against the fungal extracts (pigments) were tested and the MIC values were

measured by the microdilution method with the colorimetric indicator Alamar Blue (Invitrogen) in 96 wells microplates. The pigment extracts were added to the microplates up to 10 mg/mL (1% extract) following serial dilutions in the 9 successive wells (43).

Thin layer chromatograpy and bioautography: For the bioautography assay, *Aspergillus* and *Penicillium* were cultivated in YES medium at 25°C for seven days and extracted as previously described (44). The antimicrobial activity was determined by visualization of the inhibition area (44-46).

Bioassay with Artemia salina: The bioassays with *A. salina* were carried out following the modified Meyer et al. (47) method. *A. saline* eggs (60 mg) were added to Petri plates (90 mm x 10 mm) containing 30 mL of 3.5% (m/v) natural sea salt mix (Oceanic). For larvae outbreak, the plate was incubated at 25°C/48 hours, under constant brightness. The toxicity assay was carried out in multiwell plates (6x4), in triplicate. Each well was filled with 1800 µL of aqueous solution of 3.5% (m/v) sea salt, 10 larvae of *A. salina* and 20 µL of ethyl acetate extract diluted in dimethyl sulfoxide (DMSO) (concentration of 30 mg/mL), not exceeding 2000 µL of the total volume. In the control group, 20 µL of DMSO was used as substitute for the organic extract. After 24 hours the living and dead larvae were quantified. The degree of toxicity of the extracts was expressed in percent mortality and classified according to Harwig and Scott (48).

Statistical analysis: The data obtained were submitted to a descriptive statistical analysis using Microsoft Excel version 7.0.

Results and Discussion

Preservation in sterilized distilled water (49) became an option in Cultures DPUA Collection, being a simple method that provides the maintenance of the fungal cultures for long periods, preventing morpho-physiological modifications (50,51). The viability tests of the 30 cultures of *Aspergillus* and 30 of *Penicillium* strains, all preserved in sterilized distilled water from which over 90% were recovered in both cases. The presence of contaminants was observed in 3.3% of *Aspergillus, w*hile the phenomenon of pleomorphisms was only detected in 1.7% of *Aspergillus* and 5.0% of *Penicillium*. Morphologic alterations observed were reverted in the subcultures carried out in glycosated broth, consecutively in CYA medium. These data corroborate with the data obtained by Rodrigues et al. (52), Bueno and Gallardo (51) and Ulloa and Hamlin (53) with filamentous fungi. These results showed that the preservation process at the Cultures DPUA Collection of the Parasitology Department of the Federal University of Amazonas–UFAM was extremely efficient.

Beyond these factors, it is known that in microorganism diversity no standard technique exists that is capable to preserve them in an appropriate and widespread way (54). At this stage it is important to point out that the culture collections are conservation centers whose collection is constituted by live microorganisms, of interest for several scientific branches, besides being used in several biotechnological processes, under conditions that are established to preserve the species, in a way to maintain their vitality, specificity, activity and their immunogenic and other properties, in *ex situ* conditions (35,55). These aspects were well proven in this work.

The analysis of the cultures and of the microcultures obtained in CYA medium revealed expression of the characteristics of the *Aspergillus* and *Penicillium* genera, based on the growth rate, colony morphology, microstrutures and other characteristics compared to the specialized literature classification key (37,38,40,41) (data not shown).

The pre-screening in solid media of *Aspergillus* and *Penicillium* strains demonstrated that, among the 54 viable cultures, 13% (7 species) of the *Aspergillus* strains and 22% (12 species) of *Penicillium* strain showed pigments presence in CYA and YES media (data not

shown). The results in YES medium showed that, among *Aspergillus,* the brown pigment prevailed in relation to the others (yellow and red), the latter only being detected in *A. sparsus* DPUA 1542 cultured in CYA medium.

Table 1 summarizes the results of the *in vitro* assay of the antimicrobial activity of *Aspergillus* (n = 7) and *Penicillium* (n = 12) cultures producing soluble pigments in YES medium.

Considering the halo average diameter tests for agar diffusion, the antimicrobial activity was classified as low activity (halo = 6.0 mm to 7. mm); moderate activity (halo =8.0 to 9.9 mm) and high activity (halo = 10.0 mm). The pigments of *A. sclerotiorum* DPUA 585 and *P. simplicissimum* DPUA 1379 exhibited a high affectivity to four of the tested microorganisms. Due to these results these two microorganism were selected to attempt pigment structure

Table 1. Antimicrobial activity of 19 *Aspergillus* and *Penicillium* cultures producing colorant, against five different microorganisms

Species	**Antimicrobial activity**				
	Ca	***Cl***	***Ec***	***Ms***	***Sa***
A. carneus DPUA 1290	R	R	R	[2]S16	R
A. sclerotiorum DPUA 585	[4]S9	R	[2]S9	[1]S8	[1]S8
A. sparsus DPUA 1542	R	R	R	R	R
A. sydowii DPUA 792	R	R	R	[1]S10	[1]S8
A. sydowii DPUA 796	R	R	R	R	2S8
A. terricola 1237	[3]S12	R	R	[2]S6	[2]S8
A. terricola var. American DPUA 1272	R	R	R	R	R
P. glabrum DPUA 1435	R	[3]S11	R	R	R
P. janczewskii DPUA 304	R	R	R	R	[2]S6
P. janthinellum DPUA 1381	R	R	R	R	[2]S11
P. melinii DPUA 1391	R	[4]S26	R	[2]S11	[2]S12
P. miczynskii DPUA 1406	R	[4]S31	R	[1]S12	[2]S9
P. montanenses DPUA 1533	R	R	R	[1]S11	R
P. paxilli DPUA 938	R	R	R	[1]S8	[2]S7
P. puberulum DPUA 1146	R	R	R	R	[2]S10
P. purpurogenum DPUA 1275	[3]S11	[3]S13	R	R	R
P. purpurogenum URM 5121	R	[3]S9	R	R	R
P. simplicissimum DPUA 1379	R	[4]S13	[2]S7	[2]S14	[2]S7
P. steckii DPUA 306	R	R	R	[2]S10	R

[1]Bacteriostatic, [2]Bactericide, [3]Fungistatic, [4]Fungicide, Ca = *Candida albicans* DPUA 1336, Cl = *Cryptococcus laurentii* DPUA 1501, Ec = *Escherichia coli* CCT 0547, Ms = *Mycobacterium smegmatis* PDUFPE-71, Sa = *Staphylococcus aureus* CCT 1352, R= resistant (there was no development of the inhibition halo), S=sensible (there was development of the inhibition halo) in mm, including the proper disk of 6 mm

characterization. From all the test-microorganisms, *C. laurentii* DPUA 1501 was the most sensitive yeast to the pigments of *P. miczynskii* DPUA 1406 (halo = 31 mm) and *P. melinii* DPUA 1391 (halo = 26 mm), thus, classified as high activity species. On the contrary, *S. aureus* CCT 1352 and *M. smegmatis* PDUFPE-71 demonstrated less sensitivity to the pigments of *P. janczewskii* DPUA 304 and *A. terricola* 1237; all exhibited inhibition areas of 6 mm, being classified as low activity species. *S. aureus* CCT 1352 was shown to be sensitive to the pigments from *A. sclerotiorum* DPUA 585 (halo = 8 mm), *A. sydowii* DPUA 792 (halo = 8 mm), *A. sydowii* DPUA 796 (halo = 8 mm), *A. terricola* 1237 (halo = 8 mm) and *P. miczynskii* DPUA 1406 (halo = 9 mm), though being considered species of moderate activity.

Related to the resistance test microorganisms, the *E. coli* CCT 0547 and *C. albicans* DPUA 1336 were the most resistant to the fungal pigments under the experimental conditions. The results demonstrated also that the pigments from *A. sparsus* DPUA 1542 and *A. terricola var. American* DPUA 1272 did not exhibit antimicrobial action against the test-microorganisms (Table 1).

Table 2. Antimicrobial activity for the bioautography technique of *Aspergillus* and *Penicillium* cultures storage in sterilized distilled water of the bioactive colorants (R_f by thin chromatography).

Species	R_f	Colour[a] (λ = 366 nm)	Antimicrobial activity			
			Ca	*Cl*	*Ms*	*Sa*
A. carneus DPUA 1290	0.8	Blue	R	R	S	R
A. sydowii DPUA 792	0.8	Green	R	R	S	R
	0.6	Green	R	R	S	R
	0.4	Blue	R	R	S	R
P. glabrum DPUA 1435	0.7	Blue	R	S	R	R
	0.6	Green	R	S	R	R
P. janthinellum DPUA 1381	0.8	Green	R	R	R	S
P. melinii DPUA 1391	0.8	Blue	R	S	S	S
	0.6	Green	R	S	S	S
	0.4	Green	R	S	S	S
P. miczynskii DPUA 1406	0.8	Blue	R	S	S	R
	0.6	Green	R	S	S	R
P. montanenses DPUA 1533	0.7	Green	R	R	S	R
P. purpurogenum DPUA 1275	0.7	Green	S	S	R	R
	0.5	Blue	S	S	R	R
P. simplicissimum DPUA 1379	0.7	Yellow-	R	S	S	R
	0.6	Green	R	S	S	R
		Blue	R	S	S	R
P. steckii DPUA 306	0.7	Green	R	R	S	R
A. sclerotiorum DPUA 585	0.5	Green	S	S	S	S

Ca = Candida albicans DPUA 1336*, Cl = Cryptococcus laurentii* DPUA 1501*, Ms = Mycobacterium smegmatis* PDUFPE-71*, Sa = Staphylococcus aureus* CCT 1352, R= resistant (there was no development of the inhibition halo), S= sensible (there was development of the inhibition halo) .

[a] Spot fluorescence at $\lambda_{exc.}$ 366 nm.

On the basis of the antimicrobial activity classification for the bioautography tests only 10 species were selected, including two *Aspergillus* and eight *Penicillium* species, all of which showed high antimicrobial activity (halo = 10.0 mm) (Table 2). The results of bioautography assays revealed the presence of bioactive compounds in all the fractions obtained after extraction with ethyl acetate with at least one of the test-microorganisms of the cultures obtained from YES medium. The test microorganisms, C. *laurentii* DPUA 1501 and *M. smegmatis* PDUFPE-71 were the most sensitive to bioactive components from the species of *Penicillium* and *Aspergillus,* respectively (Table 2).

A different result was observed with *C. albicans* DPUA 1336, test microorganisms that exhibited greater resistance to the compositions of the bioactive compound. The sensitivity was only certain in the biocompounds produced by *P. purpurogenum* DPUA1275 (Rf 0.5 and 0.7*). S. aureus* CCT 1352 was sensitive to the representative bioactives detected in the extracts of *P. janthinellum* DPUA 1381 (Rf 0.8), *P. melinii* DPUA 1391 (R_F 0.4, Rf 0.6 and R_F 0.8) and A. *sclerotiorum* DPUA 585 (R_F 0.5) (Table 2).In the minimum inhibitory concentration by the microdilution method three species of fungi were sensitive: *A. sclerotiorum* DPUA 585*, P. melinii* DPUA 1391 and *P. simplicissimum* DPUA 1379. The MIC values for *E. coli* were around 2.5 to 5 mg/mL and *M. smegmatis* was sensitive from 0.31 to 2.5 mg/mL. However, *S. aureus* demonstrated sensitivity to 5 to 10 mg/mL. These data showed the inhibitory potentiality of the pigments from *P. melinii* and *P. simplicissimum* against *M. smegmatis* and *E. coli*, respectively. The yeast were resistant to all the pigments tested (10 mg/mL) (Table 3).

Table 3. Minimum Inhibitory Concentration (MIC) by the Microdilusion Colorimetric Method by Alamar Blue

Species	Crude extracts	MIC (mg/mL)
E.coli	*A. sclerotiorum DPUA 585*	5.0 (0.50%)
	P. melinii DPUA 1391	5.0 (0.50%)
	P. simplicissimum DPUA 1379	2.5 (0.25%)
M. smegmatis	*A. sclerotiorum DPUA 585*	2.5 (0.25%)
	P. melinii DPUA 1391	0,3 (0.03%)
	P. simplicissimum DPUA 1379	2.5 (0.25%)
S. aureus	*A. sclerotiorum DPUA 585*	5.0 (0.50%)
	P. melinii DPUA 1391	10.0 (1.00%)
	P. simplicissimum DPUA 1379	5.0 (0.50%)
C. albicans	*A. sclerotiorum DPUA 585*	R
	P. melinii DPUA 1391	R
	P. simplicissimum DPUA 1379	R
C. laurentii	*A. sclerotiorum DPUA 585*	R
	P. melinii DPUA 1391	R
	P. simplicissimum DPUA 1379	R

R = Resistant

Results related to toxicity of the pigments from *Aspergillus* and *Penicillium* against *Artemia salina* larvae are exhibited in Table 4. In the experimental conditions, the ethyl acetate extracts from the *Aspergillus* and *Penicillium* cultures expressed different toxicity degrees when tested with A. *saline.* The toxic action of these extracts was expressed based on the classification done by Harwig and Scott (48) in microbial extracts as: not toxic (0 to 9%); slightly toxic (10 to 49%); toxic (50 to 89%) and very toxic (90 to 100%). In this way six ethyl acetate extracts were active and classified as not toxic or slightly toxic.

Table 4 demonstrates quantitatively the fungi classification in agreement with the toxicity level. The slightly toxic ones were P. *simplicissimum* DPUA 1379 and *P. janczewskii* DPUA 304, which promoted the largest rates of mortality of the *Artemia* larvas (26.7% and 20.0%, respectively*).* It is interesting to mention that in the *in vitro* assays of the antimicrobial activity, *P. simplicissimum* DPUA 1379 presented the larger spectrum, being toxic against four of the test-microorganisms (Table 2*).* The others (17 fungi) classified as non toxic were A. *sclerotiorum* DPUA 585*, A. sydowii* DPUA 796*, A. terricola* DPUA 1237*, A. terricola var. American* DPUA 1272, *A. carneus* DPUA 1290*, A. sparsus* DPUA 1542*, A. sydowii* DPUA 792*, P. glabrum* DPUA 1435*, P. janthinellum* DPUA 1381*, P. melinii* DPUA 1391, *P. miczynskii* DPUA 1406*, P. montanenses* DPUA 1533*, P. paxilli DPUA 938, P. puberulum* DPUA 1146*, P. purpurogenum* DPUA 1275*, P. purpurogenum* DPUA 1543 and *P. steckii* DPUA 306.

Table 4. Percentage of mortality rate of the *A. salina* larvae and the toxicity level of the ethyl acetate extracts from the *Aspergillus* e *Penicillium* species

Toxicity level	Species	Mortality (%)
Slightly toxic	*P. simplicissimum* DPUA 1379	26.7
	P. janczewskii DPUA 304	20.0
Non toxic	*A. sclerotiorum* DPUA 585	6.67
	A. sydowii DPUA 796	
	A. terricola DPUA 1237	3.33
	A. terricola var. *americana* DPUA 1272	
	A. carneus DPUA 1290	0
	A. sparsus	
	A. sydowii DPUA 792	
	P. glabrum DPUA 1435	
	P. janthinellum DPUA 1381	
	P. melinii DPUA 1391	
	P. miczynskii DPUA 1406	
	P. montanenses DPUA 1533	
	P. paxilli DPUA 938	
	P. puberulum DPUA 1146	
	P..purpurogenum DPUA 1275	
	P. purpurogenum URM 5121	
	P. steckii DPUA 306	

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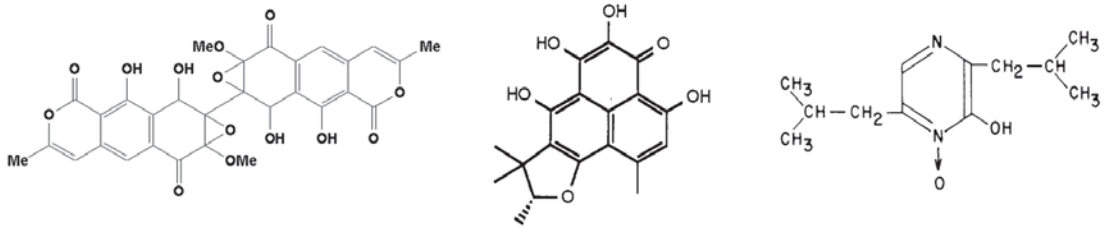
These data are different from those obtained by Sallenave-Namont et al. (56), accomplished with marine anamorph fungi. With this test 14% of *Penicillium spp.* and 15% of the *Aspergillus spp*. were classified as highly toxic. The observed level of toxicity of *Penicillium* and *Aspergillus* in our research probably are associated with the origin of microorganisms, since they are terrestrial fungi under different methods of preservation from 1 to 16 years.

From the strains non–toxic to *Artemia salina* it is worth to mention that *A. sclerotiorum* DPUA 585 and *P. melinii* DPUA 1391, which were active against 4 and 3 microorganisms (Table 4) and had no toxicity to *A. salina,* together with *P. simplicissimum* DPUA 1379, which exhibited a slight toxicity and high antimicrobial activity, were selected for an attempt to characterize the pigment structures. *P. miczynskii* DPUA 1406 that produced two pigments of blue and green fluorescence and both were no non-toxic to *A. salina* are now under analysis of its structures. It is known the production of a deep yellow-gold or yellow-brown in CYA medium by *P. miczynskii* (12).

Among *Penicillium* the yellow pigment was of greater occurrence, in both culture media, also being observed red, brown and lilac pigments. The production of these soluble pigments in the fungi culture occurred due to the influence of the composition of the culture medium, such as carbon and nitrogen sources and pH changes (57). The yellow one was produced by *P. simplicissimum DPUA 1379*, cultured in CYA medium. It is known from the literature that the main pigment from this strain is xanthoepocin (Fig.2), with antibiotic activity (58). In our strain the same color was observed (main peak at R_F 0.7 with 6:4 v/v ethylacetate:hexane and yellow-green fluorescence, mass spectrometry analyses gave the molar mass of xanthoepocin, among others components (not shown). *Penicillium melinii* DPUA-1391 also produced a yellow extract (R_F 0.6 in 6:4 and ethylacetate:hexane with a green fluorescence). It is known that *Penicillium melinii* (formerly *Penicillium atrovenetum)* produced the yellow pigment atrovenetin (Fig.2) (59) with antibiotic activity (60) and excellent antioxidant properties (61). Mass spectrometry analyses of various extracts gave the molar mass of atrovenetin, among other components. *Aspergillus sclerotiorum* DPUA-585 produced a yellow-green pigment. Probably is a similar pigment as formed from *Aspergillus sclerotiorum Huber* (CBS 549.65 strain), that produces neoaspergillic acid (Fig.2) as a yellow pigment (62) with antibiotic activity (63). This yellow pigment, when treated with ferric chloride, gives a red pigment, presumably ferrineo-aspergillin, which is red pigment. Further work is in progress in order to chemically characterize all these non-toxic pigments by NMR, FTIR and MS techniques.

Penicillium melinii was cultivated in a YES medium in a preliminary process in a pilot scale in order to study the economical feasibility of the atrovenetin production from our Amazonian strain. The preliminary study showed a production



Xanthoepocin Atrovenetin Neoaspergillic acid

Fig. 2. Chemical Structures of pigments

of atrovenetin around 1.5 mg/mL of culture medium at 25°C for 7 days. When *Penicillium atrovenetum* strain S.M. 683 was cultivated on Czapek-Dox glucose solution for 21 days at 24°C a residual yellow-brown coloring matter was purified gave around 0.7 mg/ml of a crystalline atrovenetin (64). This shows that the Amazonian strain apparently is more efficient than the previous process studied by other strain and probably might be economical feasible. Optimization of the process is under studying.

Conclusion

This pioneering investigation on pigments from *Aspergillus* and *Penicillium* strains from the Amazon forest, identifying the antimicrobial activity and the toxic action of pigment is unique. This is an important step for a feasible application of these renewable materials in the pharmaceutical and nutritional industries in a friendly and sustainable use of Brazilian resources.

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Oral immunization of Birds Against Recombinant Eimera Antigens: An Approach for Vaccinating Poultry Birds Against Coccidiosis

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Abstract

Coccidiosis is an economically important disease worldwide and is caused by the protozoan of genus *Eimeria*. In the present study we report transient expression of two coccidial antigens EtMIC1 and EtMIC2 as His_6-tagged fusion proteins in plants. Fourteen day old chickens were immunized orally with formulation containing purified recombinant antigens in combination with mucosal adjuvants like Cholera toxin-B and mineral oil adjuvant. Immunogenicity of the two formulations were evaluated in birds by estimating humoral as well as cell mediate immune response. Birds immunized with both antigens in combination with CTB as adjuvant has shown a maximum of 920 (±500) serum antibody titer against EtMIC2. The combination of oil adjuvant with antigens has shown a maximum of 720 (±580) serum antibody titer against EtMIC2. There was an average increase of about 250 pg/ml and 200 pg/ml of IFN-γ levels was measured from splenocytes induced with EtMIC2 protein. Our results indicate that the combination of plant expressed antigens adjuvanted with CTB had enhanced humoral and as well as CMI immune response in the immunized birds. Also, our results suggest that EtMIC2 protein is better immunogen compared with EtMIC1.

Keywords: Plant expressed EtMIC1 and EtMIC2, Coccidial antigens, Cholera toxin-B, mucosal immunity.

Introduction

Eimeria tenella is one of the seven species that causes the intestinal disease in chicken, which is a significant economic problem in poultry world wide. It is estimated that annual loss in poultry due to coccidiosis is more than three billion US dollars (1). The disease is spread via feco-oral route. The parasite attaches and invades into host gut epithelial cells using the proteins secreted from apical tips of sporozoite. Microneme proteins that are secreted from the apical tip facilitate the parasite invasion (2). By targeting microneme proteins as vaccine candidate many investigators have been successful in blocking the parasite invasion into epithelium (3, 4). Cholera toxin is a potent mucosal adjuvant for enteric immunization. Several studies suggest that recombinant cholera toxin B subunit when conjugated to various proteins, significantly increases the ability of these proteins to induce immune response after oral administration (5). Fabienne Girard et al investigated the adjuvant effect of cholera toxin on the intestinal and systemic immune systems of chickens (6) and Furthermore, Hyun et al. (7)

demonstrated that the binding of cholera toxin on isolated chick intestinal epithelial cells is mediated via ganglioside GM1.

Plant-based vaccines have several advantages such as ease of expression, safety, etc. which has attracted attention from investigator to exploit their utility in heterologous protein expression (8, 9). They are unlikely to be contaminated with animal pathogens, a persistent problem with tissue culture-based vaccines (10). Plant produced recombinant microneme proteins when administered parenterally in chicken were found safe, well tolerated, immunogenic and highly efficacious against homologous challenge (11). However, oral vaccines are easier to administer in largescale. In the present study we report transient expression of the microneme proteins, EtMIC1, EtMIC2 from *Eimeria tenella* as His_6-tagged fusion proteins, in tobacco using Agro-infiltration and evaluate these proteins in eliciting both humoral and cell mediated immune response in chickens when administered via oral route. Plant produced antigens were immunized in chicken in combination with mucosal adjuvants like recombinant Cholera Toxin-B (CTB) and mineral oil adjuvant (MONTANIDE IMS 1313 N VG PR).

Materials and Methods

Birds: Day-old, coccidiosis free, male White Leghorn layer chickens (commercial breed—BV 300) were obtained from Sri Venkateswara Hatcheries (Hyderabad, India) and reared in clean brooder cages. The birds were provided with coccidiostat-free feed and water *ad libidum*. Birds were shifted to animal containment facility prior to live challenge using sporulated oocysts.

Tobacco plant: *Nicotiana tabacum*, cultivar Petit Havana SR1, was cultivated in the greenhouse using vermiculate peat moss mixture. Leaves from 4-6 weeks old plants (5-6 leaf stage) were used for vacuum infiltration.

Adjuvant: Mineral oil based adjuvant MONTANIDE IMS 1313 N VG PR, an oral adjuvant was procured from SEPPIC, France.

Cloning and expression of Cholera Toxin-B in E.coli: The Cholera Toxin-B gene was constructed through Splice overlap extension PCR. Twelve overlapping forward primers (CTF1, CTF2, CTF3, CTF4, CTF5, CTF6) and reverse primers (CTR1, CTR2, CTR3, CTR4, CTR5, CTR6) were synthesized according to the CTB gene sequence (Table-1). The assembly of full length CTB gene was performed in a two step PCR. All the reactions were carried out using the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1min, annealing for 1 min at 60°C and extension at 72°C for 1 min, then 72°C for 10 min.

The SOE-PCR was performed by assembling the following overlapping set of primers CTF1, CTF2, CTF3, CTR1, CTR2 and CTR3 to generate the first fragment (1) of size 210bp. The second fragment (2) of size 198bp was generated by assembling the following sets of overlapping primers CTF4, CTF5, CTF6, CTR4, CTR5 and CTR6. Finally the full length CTB gene was developed by fusing the above two fragments (1 and 2) by using forward primer CTF1 and the reverse primer CTR6.

The SOE-PCR derived full length CTB coding sequence was cloned into bacterial expression vector pET-28a between *Eco*RI and *Not*I restriction enzyme sites. The construct was transformed into BL21-DE3 *E. coli* strain and the transformants were screened on kanamycin (50μg/ml) selective plates. *E. coli* cells harboring expression vector pET-28a-CTB were grown in LB medium. To induce expression IPTG (Genie, Bangalore) was added to a final concentration of 1mM and the culture was incubated at 28°C for 4 h. The cells were harvested and His-tagged CTB fusion protein was purified over Ni-NTA matrix.

Cloning of EtMIC1 and EtMIC2 genes into plant expression vector: The EtMIC1 gene was PCR amplified from the EtMIC1-pET28 vector (3) using forward primer 5'ATCGCCATGG AATGGCGCCCCTTCCTCGGCG3' and reverse

primer 5'GCGGCCGCG GATGCCCACAT CTCTGATTGTT3'. The amplified product was cloned into plant expression vector pTRA-ERH in between *Nco*I and *Not*I restriction enzyme sites. Cloning of EtMIC2 gene is described elsewhere (11). pTRA EtMIC1 and pTRAEtMIC2, were sequenced from vector back bone using the following pTRA sequencing primers, forward primer 5'AAGACCCTTCCTCTATATAAG3'and reverse primer 5'GAGCGAAACCCTATAAGA ACC3' to confirm the presence of the insert.

Agrobacterium tumefaciens strain GV3101 was transformed with the recombinant constructs using electroporation. Transformed *Agrobacterium* cells were plated on YEB plates [0.5% (w/v) peptone, 0.5% (w/v) beef extract, 0.5% (w/v) sucrose, 0.1% (w/v) yeast extract, 2mM $MgSO_4$, 1.5% agar, pH- 7.4] containing 100µg/ml carbenicillin and 25 µg/ml rifampicin and incubated for 72 hours at 28ºC. The *Agrobacterium* colonies were screened using gene specific PCR to identify the recombinant clones.

Agrobacterium mediated Transient expression and purification of EtMIC1 and EtMIC2 proteins: The recombinant *Agrobacterium* clones were grown overnight at 28ºC in YEB broth containing 100µg/ml carbenicillin and 25 µg/ml rifampacin. Bacterial cells were harvested by centrifuging the cells at 6000 rpm. The *Agrobacterium* pellet was resuspended in induction medium [YEB medium adjusted to pH-5.6 supplemented with 20µM acetosyringone, 10mM 2-N-morpholino-ethane-sulphonic acid (MES)] and incubated at 28ºC for 16 hours. The bacterial cells were harvested by centrifugation and the cells were resuspended in MMA medium containing [4.6 g/l Murashige and Skoog basal medium, 2% (w/v) sucrose, 10mM MES, pH- 5.6] 200µM acetosyringone. The optical density (OD_{600}) of the suspension was adjusted to 2.0 using the MMA medium and the bacteria were incubated for 2 hours at room temperature (24±4°C). Vacuum infiltration of the resuspended culture was carried out as described by Kapila et al (12). The infiltrated leaves were incubated at 15°C for 64 hours under 16 hours light and 8 hours dark photoperiod. The leaves were then stored at -80°C until further use. Soluble protein was extracted from infiltrated leaves as described earlier (11). The recombinant proteins were purified over Ni-NTA matrix. The purified protein was dialyzed extensively against PBS to remove imidazole. Yield of the affinity-purified protein was estimated using Bicinchoninic Acid kit (Sigma–Aldrich, USA). The purified protein was stored in aliquots at -20ºC until further use.

SDS-PAGE analysis and Immunobloting of all recombinant proteins: Purified recombinant proteins were resolved on 12% SDS-PAGE under reducing conditions and the Protein were visualized after staining with Coomassie brilliant blue. The protein was also electro-blotted on to PVDF membrane (Hybond-P; GE-Healthcare, USA) and the membrane was blocked at room temperature with 3% (w/v) skimmed milk powder dissolved in PBS. The blots were probed using anti-His_5 monoclonal antibody conjugated to horse-radish peroxidase (1:3000 dilution; Qiagen, Germany).

Immunization: Birds were divided into four groups containing 14 birds each. To immunize birds via oral route, known amount of antigen was mixed along with layer mash and given to the birds. Birds in group-I were immunized with a combination of antigen containing 100µg of plant expressed EtMIC1 and EtMIC2 each adjuvanted with 50µg of *E. coli* expressed CTB. Similarly birds in group-II were immunized with a of 100µg of plant expressed EtMIC1 and EtMIC2 in adjuvanted with equal volume of MONTANIDE IMS 1313 N VG PR. Birds in group-III were immunized with a mixture of 100µg of EtMIC1 and EtMIC2 without any adjuvant. Birds in group-IV were 'unimmunized' control. The immunization schedule consisted of one primary dose on 7^{th} day and two booster doses on 14^{th} and 21^{st} days.

(i) Humoral immune response: Birds were bled prior to each immunization and also on 28^{th} day post primary immunization. Serum antibody titers

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against EtMIC1 and EtMIC2 were measured using an indirect ELISA. Maxi-sorp ELISA plates were coated with *E. coli* expressed EtMIC1 or EtMIC2 protein to assess specific antibody titers in immunized chicken sera. Titers in the serum was defined as maximum sera dilution showing OD_{450} greater than mean (+3×SD) of pre-immune sera (11). Statistical analysis were performed using the Origin Pro (version-8.0) software and the difference in mean was subjected to one way Anova.

(ii) Evaluation of cell mediated immune response: IFN-γ expression level was evaluated in orally immunized birds with respect to uninduced naïve birds. Spleens were collected after euthanizing birds on days 3, 6 post final immunization. Five birds were splenectomized on each day of sampling. Splenocytes from individual birds were cultured separately. The splenocytes cultured from the spleens of mock-immunized birds were used as negative control. The splenocytes were obtained by perfusing cell culture media (RPMI 1640; Invitrogen, USA) through the spleens. The cell counts were adjusted to 10^6 cells/ml using RPMI supplemented with 10% fetal bovine serum. One million cells were seeded per well in a 24 well tissue culture plate (Nunc, Denmark). The splenocytes were stimulated using 20 μg/ml of recombinant *E. coli* expressed EtMIC1 and EtMIC2 proteins at the time of seeding. Twenty microgram per ml of *E. coli* expressed Heat shock protein (Hsp), was used for mock stimulation while 15 μg/ml of concanavalin A (Genei, India) was used as positive control for the cytokine response in splenocytes. The splenocytes were incubated at 37°C with 5% CO_2 for 64 hours. After incubation the culture supernatant was collected 65hrs after induction and IFN-γ level in the culture supernatant were estimated using Chicken IFN-γ capture ELISA detection kit (Invitrogen). The IFN-γ levels were measured by comparing the sample OD_{450} values with the OD_{450} values of the kit standard. The results were expressed as picograms of IFN-γ per 100μl of sample.

Results and Discussion

Assembly, cloning and expression of Synthetic CTB: CTB is a part of the bacterial toxin complex along with cholera toxin A that induces diarrhea and results into symptoms produced due to the infection of *Vibrio cholera* in the human gut. However CTB is the nontoxic component of the cholera toxin whose primary

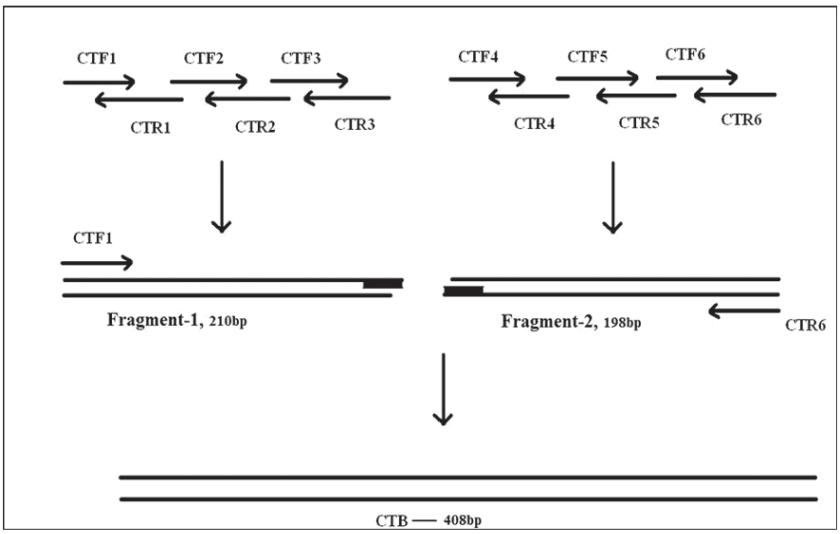

Fig.1. Schematic representation of Splice over extension PCR developed to generate full length Cholera Toxin- B gene.

function involves in formation of a pentameric structure that bind to GM1-ganglioside receptors of the gut mucosal epithelial cells and thereafter up regulate special receptor gene expression on some gut epithelial cell (13-19). The 5'-210 bp size CTB gene fragment was assembled by using three forward primers CTF1, CTF2, CTF3, that overlapped with three reverse primes CTR1, CTR2, CTR3 respectively. Similarly, the 3'-198bp CTB gene fragment was generated by overlapping forward primers CTF4, CTF5, CTF6 and reverse primers CTR4, CTR5, CTR6. The full-length, 408bp CTB-gene was assembled in the 2nd Step PCR using the PCR amplicons of the first-PCR as templates. The full-length genes were cloned into pET-28a vector and screened by restriction enzyme analysis with *Eco*RI and *Not*I restriction enzymes (Fig 1 & 2). The recombinant clones were subsequently sequence verified (data not shown).

The purified CTB protein obtained from the expression of SOE assembled gene was assayed for ability to bind GM1 gangliosides in an ELISA (data not shown). The GM1 ganglioside binding ability of the purified CTB indicates that the expressed protein is similar in its tertiary structure to the native protein. SOE-PCR aided by the new-age high fidelity polymerases is a powerful tool for assembling full-length genes of interest from synthetic oligonucleotides. This technique is increasingly being employed in molecular biology for cloning and expression of genes of interest that are either difficult to source or involve culturing potentially dangerous pathogenic organisms. Several investigators report that CTB could be used to enhance the efficacy of the potential drug proteins or peptides conjugated to it by nasal or oral administration (20, 21).

EtMIC1 & EtMIC2 gene cloned into pTRA-ERH vector and creation of Agrobacterium clone: Microneme organelles are located at the apical tip of invading stage of all apicomplexan parasites and are essential for motility of the parasite, identification and binding of the host cell–surface proteins and invasion of host cells (3). Recent studies concluded that use of these microneme proteins as subunit vaccines could block the parasite invasion into gut epithelium and helps in protection (3, 4, 11). 2.8kb size EtMIC1 gene was obtained by amplifying with gene specific

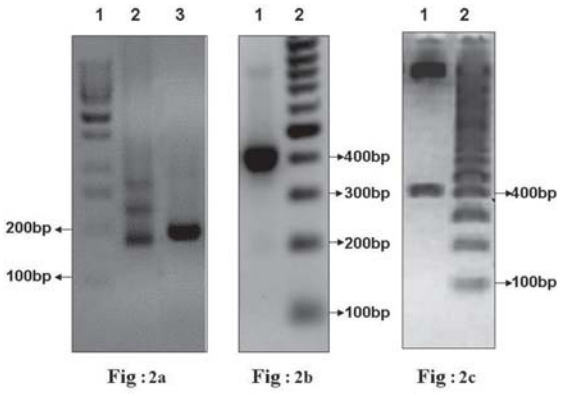

Fig.2. Agarose gel showing (**2a**) amplified Fragment 1 (lane2) of size 198bp, Fragment 2 (lane 3) of size 210 bp, (**2b**) Full length Cholera toxin-B gene (lane1) of size 408 bp, (**2c**) released product of CTB gene of size 408 bp after digestion with *Eco*RI and *Not*I enzymes.

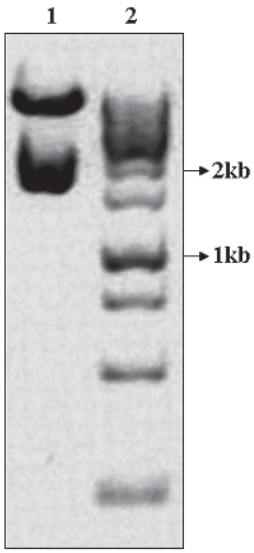

Fig.3. Agarose gel showing the released product of 2.8kb size EtMIC1 gene digested with *Nco*I and *Not*I restriction enzymes (lane-1) and Fermantas 1kb marker (lane-2) .

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primers. Cloning of EtMIC1 gene into pTRA-ERH vector was confirmed up on restriction digestion with *Nco*I and *Not*I enzymes (Fig-3). Recombinant *Agrobacterium* clones were screened using specific primers for EtMIC1 sequence, *Agrobacterium* clones harboring EtMIC1 gene produced a PCR amplification product of 2.8kb size. EtMIC2 cloning is described elsewhere (11).

Expression confirmation of recombinant proteins by immono-blot: Coccidial proteins EtMIC1, EtMIC2 were produced at a high level in *Nicotiana tabacum* Petit Havana var SR1 leaves through Agrobacterium mediated transient expression and purified using IMAC. Expression analysis showed that both the proteins were produced in detectable amounts in plant cells, but proportionally more EtMIC2 (50kDa) was expressed than EtMIC1 perhaps due to the higher molecular weight of EtMIC1 (100kDa). Similarly, CTB protein was expressed in *E. coli* (BL21DE3) and purified using IMAC. The purified recombinant EtMIC1, EtMIC2 and CTB proteins were analysed using immuno-blotting by probing the blot using anti-His_5 monoclonal antibody. A protein bands of approximately 18 kDa for CTB, 100 kDa for EtMIC1 and 55 kDa for EtMIC2 were detected in the immuno-blot (Fig-4) which corresponds to the expected size. The yield of EtMIC1 and EtMIC2 were estimated to be 25mg and 50mg/Kg fresh biomass.

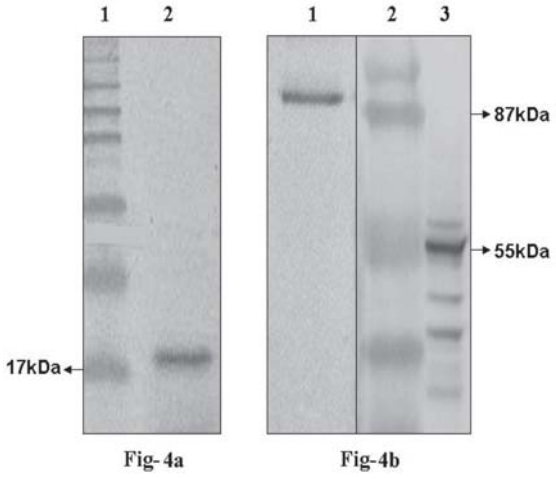

Fig. 4. (4a) Immunoblots demonstrating *E. coli* expressed Cholera Toxin-B protein band showing at ~18 kDa (lane2), (4b) Plant expressed EtMIC1 & EtMIC2 protein bands showing at 100 kDa (lane1) & 55kDa (lane 3). All blots were showing the specific reactivity against Anti-His5 HRPO conjugate monoclonal antibody. Lane 1 in (4a) is pre-stained marker (NEB), and Lane 2 in (4b) is pre-stained marker (Fermentas).

Immunization

(i) Serum antibody response in immunized birds: To test the immunogenicity of the plant derived coccidial antigens, we had earlier reported administration of plant proteins parenterally to chicken and found the chickens to be protected against homologous challenge (11). In order to explore the mass vaccination in birds the plant derived antigens were administered *ad libitum* over several feedings and both humoral and cell mediated immune response was evaluated. The antigens were adjuvanted using CTB or mineral oil adjuvant (MONTANIDE IMS 1313 N VG PR). This oil was choosen as adjuvant because Jang et al (22) had reported that the oil adjuvant was able to potentiate immune response in birds when given through oral route. The serum samples collected from the immunized birds on days 14, 21 and 28 were analyzed for the presence of the IgG antibodies. Birds in Group-I were immunized with EtMIC1, EtMIC2, in combination with CTB. Average serum antibody titers against EtMIC1 was found to be as 90 (±45) on 14th day, 125 (±54) on 21st day, and 315 (±469) on 28th day. For EtMIC2 the titers were 270 (±200) on 14th day, 560 (±263) on 21st day, and 920 (±500) on 28th day. Birds in Group-II were immunized with EtMIC1, EtMIC2, in combination with mineral oil adjuvant. Average serum antibody titers against EtMIC1 was 80 (±25) on 14th day, 120 (±100) on 21st day, and 155 (±100) on 28th day. For EtMIC2 it was 210 (±110) on 14th day, 410 (±233) on 21st day, and 720 (±580) on 28th day. Birds in Group-III were immunized with EtMIC1 and EtMIC2 alone without any adjuvant. Average serum

antibody titers against EtMIC1 was 210 (±330) on 14th day, 420 (±661) on 21st day, and 280 (±294) on 28th day. For EtMIC2 it was 110 (±54) on 14th day, 260 (±304) on 21st day, and 340 (±279) on 28th day (Fig 5 & 6).

It was observed that serum antibody response was better against both the antigens in birds that were immunized in combination with mucosal adjuvants when compared with the birds immunized without adjuvant. Furthermore, CTB could potentiate induction of higher antibody response against both antigens when compared with mineral oil adjuvant. One way analysis of variance (Anova; *p =0.05) for the serum antibody titer indicated that birds in any of the three groups had significant increase in the serum antibody titers for EtMIC1 (Figure 5). One way analysis of variance (Anova; *p =0.05) indicated that Group-I & Group-II birds had significant increase in the serum antibody titers for EtMIC2, while Group-III did not have significant increase in the serum antibody titers (Figure 6). We have earlier reported good sero-conversion with EtMIC1 administered parenterally (3). It is likely that the recombinant EtMIC1 is not effectively sampled by MALT for antigen presentation, leading to poor sero-conversion. However, we would have to investigate this hypothesis in detail to ascertain our claim.

Increasing the level of serum antibodies which are directed against parasite antigens of survival importance will likely enhance local protection against coccidiosis. Enhanced production of these antigens in combination with CTB in plants and periodically fed with supplementary diet may provide a new opportunity to utilize in increasing the local immunity, eventually reducing the economic loss due to coccidiosis. Furthermore for complex parasitic infection like coccidiosis whose treatment essentially depends on drugs immune enhancement using food plants provides a safe alternative control method.

(ii) Estimation of IFN-γ expression level from immunized birds: *E. coli* expressed recombi-

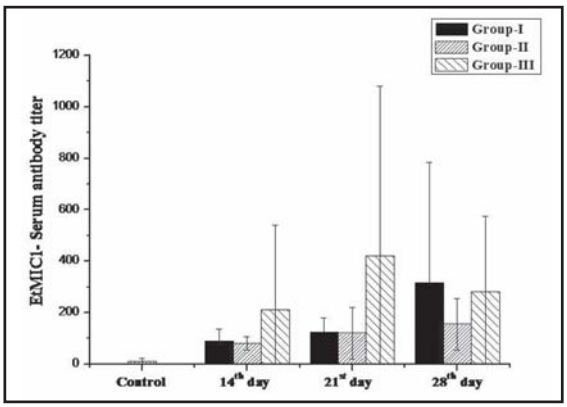

Fig. 5. EtMIC1 antibody specific ELISA titers (mean±SD) from birds immunized with plant expressed EtMIC1 & EtMIC2 in combination with oral adjuvants. The assay was performed using indirect ELISA in a Maxisorp plate coated with *E. coli* expressed EtMIC1 protein. Antibody titers in the serum were determined as maximum sera dilution showing OD_{450} greater than mean + 3×SD of pre-immune sera.

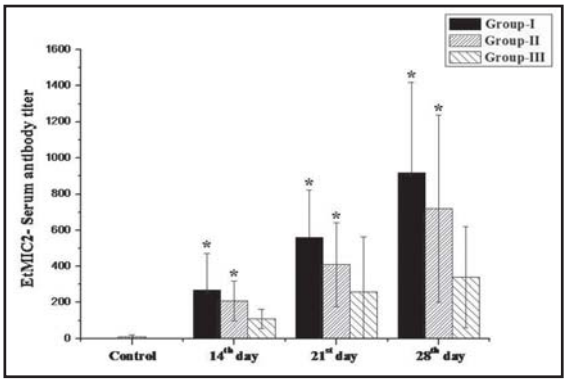

Fig. 6. EtMIC2 antibody specific ELISA titers (mean±SD) from birds immunized with plant expressed EtMIC1 & EtMIC2 in combination with oral adjuvants. The assay was performed using indirect ELISA in a Maxisorp plate coated with *E. coli* expressed EtMIC2 protein. Antibody titers in the serum were determined as maximum sera dilution showing OD_{450} greater than mean + 3×SD of pre-immune sera. The asterisks indicate significant increase in serum antibody titer in different formulation groups (*p < 0.05; N = 14).

nant EtMIC1 and EtMIC2 were used to stimulate the splenocytes. Sixty five hours after stimulation the IFN-γ levels in the culture supernatant were quantified using capture ELISA kit. There was no significant antigen specific IFN-γ response on 3rd and 6th day post final immunization in birds. The average expression of IFN-γ in birds immunized with EtMIC1, EtMIC2 and CTB on 3rd day post final immunization was found to be 250pg/ml compared to mock induced splenocytes. The average expression of IFN-γ in birds immunized with EtMIC1, EtMIC2 and MONTANIDE IMS 1313 N VG PR on 3rd day post final immunization was also found to be 200pg/ml compared to mock induced splenocytes (Fig-7). There was no detectable IFN-γ response in birds immunized with EtMIC1 and EtMIC2 alone. In contrast to our earlier work (11) IFN-γ response after oral immunization did not seem to have any significance in this study. There is very marginal IFN-γ response from oral immunized birds on 6th day and found completely no response on 9th day post final immunization. It may be reasonable to expect that humoral response at the site of infection may help reduce the disease burden in the flock. Given that the birds in poultry have a short 'shelf life' extensive CMI response may not be necessary. However, we had demonstrated that the recombinant antigens used in the present study have the ability to induce CMI response when administered parentarally.

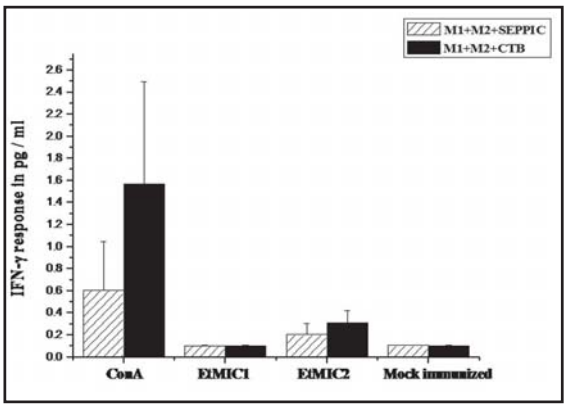

Fig. 7. IFN-γ levels quantified using capture ELISA among vaccinated birds compared to unimmunized birds on day 3 post immunization (N=5). There was an average increase of about 250pg/ml and 200pg/ml of IFN-γ levels was measured from splenocytes induced with EtMIC2 protein.

Conclusion

Poultry birds are reared under intense conditions, which reduce the scope of maintaining good hygiene. The chemo-prophylactic measures are likely to be phased out (23), coccidiosis in poultry urgently requires

Table 1. The overlapping primers employed in synthesizing full length CTB by SOE-PCR.

S.N.	Name	5'→3' Sequence
1	CTF1	ATGCGAATTCATGAATAAGGTTAAATTTTATGTGCTCTTTACCGCTCTGCTT
2	CTF2	TGCGCCGGGCTATGCTCATGGGACCCCACAGAATATTACGGATCTGTGTGCGG
3	CTF3	GCAAATCCATACACTGAATGATAAAATTTTCTCCTACACCGAAAGTTT
4	CTF4	GGCGATCATTACCTTCAAAAACGGTGCGACATTTCAAGTGGAGGTACCGGG
5	CTF5	AGCCAGAAAAAGGCCATTGAACGTATGAAGGACACTCTGCGCATTGCAT
6	CTF6	GAGAAATTGTGCGTCTGGAACAACAAAACGCCTCACGCCATTGCGGC
7	CTR1	AGCCCGGCGCACCATGGGCGCACAGAGAGCTAAGCAGAGCGGTAAAG
8	CTR2	GTATGGATTTGCGTATTGTGATATTCCGCACACAGATCCGT
9	CTR3	GAAGGTAATGATCGCCATTTCCCGTTTGCCGGCTAAACTTTCGGTGT
10	CTR4	GGCCTTTTTCTGGCTATCGATATGCTGCGATCCCGGTACCTCCACTTG
11	CTR5	CCAGACGCACAATTTCTCAACTTTTGCTTCAGTTAAGTATGCAATGCGCAG
12	CTR6	GATCGCGGCCGCGTTAGCCATTGAAATTGCCGCAATGGCGTG

alternative prophylactic measures that are safe and cost effective (24, 25, 26). Our results provide the first demonstration that a plant expressed EtMIC1 & EtMIC2 antigens in combination with CTB had effectively enhanced the humoral immune response in birds immunized via oral route. The results recapitulate that CTB is better in adjuvanting property compared to mineral oil adjuvant. It also illustrates that EtMIC2 is a better immunogen than EtMIC1 when given orally along with adjuvants. This study strengthens the case for exploiting CTB as oral adjuvant in combination with plant expressed antigens to immunize the birds against coccidiosis. Developing an oral vaccine seems a promising approach, both in terms of the logistics for large-scale vaccine delivery as well as affordability for the prophylactic vaccine administration. However, further studies are necessary to translate the proof of concept presented in this manuscript into a vaccine.

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Assessment of Allelopathic Property of *Mikania scandens* Root

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Abstract

The present study aimed the evaluation of allelopathic effect of hydroalcoholic extract from the roots of *Mikania scandens* (L.) Willd. (Asteraceae); against the germination and radicle growth of *Cicer arietinum* and *Triticum aestivum* seeds. The extract at different concentrations was incubated in controlled conditions with the surface sterilized seeds of *C. arietinum* and *T. aestivum* and observed periodically for seed germination and radicle growth to assess the allelopathic behaviour. The extract mainly at higher concentrations demonstrated promising allelopathic potential by significantly affecting seed germination and radicle elongation of both *C. arietinum* and *T. aestivum* in a concentration dependent manner. *C. arietinum* was found to be more sensitive than *T. aestivum*. The present study demonstrated remarkable allelopathic potential of *M. scandens* root against the test seeds. The effect was plausibly due to the alkaloids and polyphenols present in the root of *M. scandens*.

Keywords: Allelopathic, *Mikania scandens*, *Cicer arietinum*, *Triticum aestivum,* roots.

Introduction

Indiscriminate use of synthetic herbicides has resulted in herbicide-resistant weeds and environmental concerns about the safety of conventional synthetic herbicides. Therefore, there is need for alternative weed management systems which are less synthetic herbicide dependent or based on naturally occurring compounds (1). Allelopathy holds promise for the environmentally friendly weed management. The phenomenon of allelopathy, where a plant species chemically interferes with the germination, growth or development of other plant species has been known for over 2000 years. Allelopathy can be defined as any direct or indirect harmful or beneficial effect of one plant on another through the production of chemicals that it releases into the environment (2). In 1996, the International Allelopathy Society defined allelopathy as follows: “Any process involving secondary metabolites produced by plants, micro-organisms, viruses, and fungi that influence the growth and development of agricultural and biological systems (excluding animals), including positive and negative effects” (3). Chemicals released from plants and imposing allelopathic influences are termed allelochemicals or allelochemics. Most allelochemicals are classified as secondary plant metabolites which are biosynthetically derived from the primary metabolites of the plant (4). When susceptible plants are exposed to allelochemicals, germination, growth and development may be affected. Allelochemicals are present in several parts of plants that are known to interfere with seed germination and growth of neighbouring or successional plants by releasing allelochemicals in their environment (1, 2). The search and development of new herbicides through the identification of active compounds from allelopathic plants is an interesting research and development area.

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These compounds can be regarded as 'natural herbicides'. Several plants are reported to possess allelopathic potential and efforts have been made to apply them for weed control (5).

Mikania scandens (L.) Willd. (Asteraceae), known as climbing hemp weed in English, is a twining herbaceous climbing vine with long-petioled, opposite leaves and small homogamous flower-heads, grown abundantly throughout the plains of India and Bangladesh. Traditionally, the plant has been used for some medicinal purposes in the Indian subcontinent. Aqueous leaf extracts of this plant have been used in folk medicine to treat stomach ulcers. The plant is thought to be efficacious in the treatment of gastric problems. Traditionally its leaf juice is applied to the affected area of body in treatment of wounds and bruises. The plant is regarded as a rich source of vitamin A and C and also contains vitamin B, mikanin, friedelin, efifriedinol, and some sesquiterpene dilactones including mikanolide, dihydromikanolide, deoxymikanolide, and scandenolide. Three deterpenic acids known as kaurenic acid, butyryloxykaurenic acid, and benzoyloxykaurenic acid, stigmasterol and betasitosterin have also been isolated from this plant (6-8).

It has come to the author's notice that the rural people of Hooghly, Bardhaman, and Medinipur districts of West Bengal state of India use the young leaves of this plant in management of insect bites and stings. Previous workers reported analgesic and *in vitro* antioxidant activities of *M. scandens* leaf (9). In our previous study, we have reported neuropharmacological and allelopathic properties of *M. scandens* aerial parts (10, 11), and *in vitro* anti-inflammatory effects of aerial parts, root and flower of *M. scandens* (12, 13). The present study was conducted to assess the possible allelopathic potential of the roots from *M. scandens* against the germination and radicle growth of *Cicer arietinum* and *Triticum aestivum* seeds.

Materials and Methods

Plant material : The roots of *M. scandens* were collected during October, 2011 from Gotan region of Bardhaman district of West Bengal, India. The species was authenticated by Dr. P. Lakshminarasimhan, Scientist D, at the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal, India, and a voucher specimen (CNH/44/2011/Tech.II/476) was deposited at the Pharmacognosy Research Laboratory, Bengal School of Technology, Delhi Road, Sugandha, Hooghly 712102, India. Just after collection, the plant material was washed thoroughly with running tap water and shade dried at room temperature (24-26 ºC) and ground mechanically into a coarse powder.

Preparation of extract: The powdered plant material (50 g) was extracted with 50% aqueous ethanol (400 ml) by boiling under reflux for 90 minutes. The extract was filtered and evaporated to dryness to yield the dry extract (RMS, yield: 8.35%). The dry extract was kept in a refrigerator until use. Preliminary phytochemical studies were performed on RMS as per reported method (14).

Test samples: The test samples for allelopathic bioassay were prepared freshly from the dry extracts. Different concentrations of both the test extracts, viz., RMS (40, 20, 10, 5, 2.5, 1.25 mg/ml), were prepared by dissolving in double-distilled water immediately prior to use.

Collection and preparation of Cicer arietinum and Triticum aestivum seeds: Healthy uniform seeds of gram (*Cicer arietinum* L., family: Fabaceae) and wheat (*Triticum aestivum*, family: Poaceae) were obtained from the Agriculture Seed Store (Govt. of West Bengal) Kalyani, West Bengal, India. The seeds were soaked in distilled water for one hour. Then the seeds were surface sterilized with 70% ethanol for 2 minutes, then rinsed with double-distilled water for several times for complete removal of the sterilant.

Exposure to text samples: This procedure was performed under aseptic conditions at laminar air-flow bench. The surface sterilized seeds were placed evenly in sterilized glass Petri dishes (9 mm). Each Petri dish contained 10 seeds. Then equal volume (5 ml) of varying concentrations of the test samples were introduced into each Petri

dish. Similar volume of double distilled water was used as control. In case of wheat seeds the test liquids were decanted after 30 minutes. Then all the Petri dishes were incubated in dark at room temperature (24-26°C) for 96 h in case of gram seeds and for 48 h in case of wheat seeds. Allelopathic behaviour was evaluated by recording the number of germinated seeds and radicle length using a millimetre ruler, after 48, 72 and 96 h in case of gram seeds, and after 24 and 48 h in case of wheat seeds. The indicating parameters viz., germination percentage and percentage inhibition of radicle growth were calculated by the following formulae:

Germination percentage = Number of germinated seeds/Total number of seeds ×100

% Inhibition of radicle growth= (X–Y)/X ×100.

Where, X= Control mean radicle length and Y= Treated mean radicle length.

The extract concentration for 50% radicle length inhibition (IC_{50}) was determined by plotting percentage inhibition of radicle growth with respect to control against treatment concentration.

Statistical analysis : The data of radicle length were expressed as the mean ± standard error of mean (SEM). Same data were analyzed for statistical significance by Student's '*t*' test. *P* values less than 0.05 (*p* d" 0.05) were considered as statistically significant.

Results and Discussion

Screening of plant extracts and their fractions for their effects on seed germination of various plant species are routinely used to evaluate their alleleopathic potential (5). The present findings demonstrated negative allelopathic effects of hydroalcoholic extract of *M. scandens* root (RMS) on the germination and radicle growth of *C. arietinum* and *T. aestivum* seeds.

The results of allelopathic effect of RMS on *C. arietinum* are summarized in Tables 1 and 2. RMS at all test concentrations inhibited germination of *C. arietinum* seeds in a concentration dependent way; however, 70% seeds were found to germinate at lower concentration (1.25 mg/ml) at each time interval (Table 1). RMS remarkably inhibited radicle growth at the all test concentrations in a time and concentration dependent manner. The effects were found to be prominent and significant during the whole observation period (Table 2).

The results of allelopathic effect of RMS against *T. aestivum* are presented in Tables 3 and 4. RMS at all test concentrations inhibited germination of *T. aestivum* seeds in a concentration dependent fashion; however, no germination was observed in case of higher concentrations of RMS (20 and 40 mg/ml) during 48 h (Table 3). RMS significantly and concentration dependently inhibited radicle growth at all the test concentrations during 48 h of observation. No detectable radicle growth was observed at the higher concentrations of RMS (20 and 40 mg/ml) even up to 48 h (Table 4). The IC_{50} values of RMS for both *C. arietinum* and *T. aestivum* are summarized in Table 5.

The most frequently reported allelo-chemical-induced gross morphological effects on plants include inhibited or retarded seed germination, effects on coleoptile elongation and on radicle, shoot and root development (15). Here, germination percentage and radicle growth were recorded to monitor the allelopathic behaviour. However, in the present study radicle growth appeared to be the most sensitive parameter and IC_{50} values based on this parameter very clearly indicated the differential allelopathic effect of RMS on both test seeds (Table 5). From these values it becomes evident that *C. arietinum* was more sensitive to RMS, being effective in lower concentrations; than *T. aestivum* after 48 h.

Plants exhibit allelopathic activity due to release of allelochemicals of different chemical classes mainly polyphenolic compounds (flavonoids and tannins), cyanogenic glycosides and alkaloids (4, 16). The inhibitory effect of the

Table 1. Effect of RMS on germination percentage of *C. arietinum.*

Concentration (mg/ml)	After 48 h (%)	After 96 h (%)	After 72 h (%)
Control	100	100	100
1.25	70	70	70
2.5	50	50	60
5	30	50	50
10	0	0	0
20	0	0	0
40	0	0	0

test extract on seed germination and radicle length may be due to the presence of putative allelochemicals. Preliminary phytochemical analysis revealed the presence of alkaloids, saponins, polyphenolics and carbohydrates in RMS. Polyphenols are well known natural products reported to possess several notable biological properties (17). In the present study, allelopathic effect of RMS could be attributed to its alkaloid and polyphenol contents. The observed effect may be due to synergistic effect rather than single constituent.

From the present preliminary investigation, it can be concluded that *M. scandens* root exhibited remarkable negative allelopathic potential by significantly affecting the germination and radicle growth of both *C. arietinum* and *T. aestivum. C. arietinum* was found to be more sensitive than *T. aestivum*. To the best of our knowledge, this is the first report of allelopathic effect of *M. sandens* root. Allelopathic effects of *M. scandens* root under field conditions also need further research in pursuit of a new, effective and environment friendly natural herbicide.

Table 2. Effect of RMS on radicle growth of *C. arietinum.*

Concentration (mg/ml)	After 48 h		After 72 h		After 96 h	
	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length(mm)§	%Inhibition of of radicle growth	Radicle length (mm)§	% Inhibition of radicle growth
Control	19.20±2.5	-	39.50±5.43	-	60.0±5.45	-
1.25	6.52±1.43*	66.04	8.50±2.45*	78.48	10.86±3.15*	81.90
2.5	5.57±1.26*	70.98	7.64±3.08*	80.66	8.43±3.21*	85.95
5	2.06±0.81*	89.27	3.83±0.79*	90.30	4.61±0.80*	92.32
10	0	100	0	100	0	100
20	0	100	0	100	0	100
40	0	100	0	100	0	100

§Data are expressed as mean ± SEM. *$p < 0.001$ compared with control.

Table 3. Effect of RMS on germination percentage of *T. aestivum.*

Concentration (mg/ml)	After 24 h (%)	After 48 h (%)
Control	60	90
1.25	30	40
2.5	20	30
5	0	20
10	0	10
20	0	0
40	0	0

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Table 4. Effect of RMS on radicle growth of *T. aestivum.*

Concentration (mg/ml)	After 24 h		After 48 h	
	Radicle length (mm)§	% Inhibition of radicle growth	Radicle length(mm)§	%Inhibition of of radicle growth
Control	2.17± 0.30	-	2.67 ± 0.47	-
1.25	1.20 ± 0.31£	47.70	1.66 ± 0.56¶	37.83
2.5	1.0 ± 0.20£	53.91	1.30 ± 0.44¶	51.31
5	0	100	1.0 ± 0.28£	62.55
10	0	100	0	100
20	0	100	0	100
40	0	100	0	100

§Data are expressed as mean ± SEM. £ $p< 0.02$, ¶ $p< 0.05$ compared with control.

Table 5. IC_{50} values of RMS on radicle growth of *C. arietinum* and *T. aestivum.*

Treatment time	IC_{50} (mg/ml)	
	C. arietinum	*T. aestivum*
After 24 h	-	1.65
After 48 h	0.95	2.18
After 72 h	0.80	-
After 96 h	0.76	-

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Estimation of Biochemical Activities of Microbial Load Isolated from the Frozen Semen of HF and HF Crossbred Cattle Bulls

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Abstract

Total 30 French mini straws (0.25ml) of frozen semen of HF and HF crossbred cattle bulls were randomly collected from one of the frozen semen banks for evaluation of microbial load using the standard plate count (SPC) method using nutrient agar plate. These plates were incubated at 37°C for 72 hrs and examined for growth. The average colony count was calculated and bacteria were also identified as Gram positive and Gram negative. A total of 10 biochemical tests were performed to characterize the isolates. Antibiotic sensitivity test was also performed to test the sensitivity against Ampicillin, Erythromycin, Gentamycin and Spectinomycin. The results indicate that eleven samples out of 30 (36.6%) were found positive for various bacterial isolates. In most samples the microbial load was found below the Bureau of Indian Standard (BIS). However one sample (3.33%) was found with fungal infection. Both the Gram positive and Gram negative bacteria were found in these samples. Results of biochemical properties of bacterial isolates are summarized. Each isolates are varying in their biochemical profile. The results of antibiotic sensitivity pattern in bacterial isolates were also summarized. All bacterial isolates exhibited variable pattern against Ampicillin, Erythromycin, Gentamycin and Spectinomycin. The concentrations of antibiotics were 10µg, 10µg, 10µg and 100 µg respectively. Among all these antibiotics all bacterial isolates are resistance against Ampicillin. The article describes detailed investigation of microbial load in frozen semen of HF and HF crossbred cattle bulls

Keywords: Holstein Cattle bull, frozen semen, microbial load, Biochemical tests, microorganism,

Introduction

Holstein cattle are a breed of cattle known today as the world's highest production dairy animal. India is using HF cattle bulls for Artificial Insemination (AI) for the improvement of Indian cattle by way of crossbreeding. The success of AI programme depends on quality semen production. The bacterial contaminants of semen have been a major concern for most semen production laboratories as it adversely affects the semen quality (1) and hence the subsequent fertility (2, 3). Macrophages and polymorpho-nuclear granulocytes, which form the first line of defense against microorganisms, produce reactive oxygen species (ROS) to kill these microorganisms. ROS, however, is also released outside these cells and may react with the molecules and cells such as spermatozoa in their vicinity (4). The cellular antioxidants, present mostly in the cytoplasm, are scanty and inadequate to counteract this ROS because the sperm cell cytoplasm is very small (20 mm^3) and is mostly distributed on the midpiece (5). The bacterial load in the semen samples is estimated by standard plate count (SPC) method. Few bacteria of semen survive at –196 °C in liquid nitrogen and acquire a certain level of resistance to antibiotics (6) and account for the

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contamination of approximately 50 % of frozen semen samples (7). The bacterial contaminants of the semen have been classified as pathogenic, potentially pathogenic or non-pathogenic. Gram's staining is very important method of differentiating bacterial species into two large groups; Gram positive and Gram negative, based on the chemical and physical properties of their cell wall (8). Different biochemical tests are also used as additional tools to identify and characterize the bacterial isolates. In vitro sensitivity to different antibiotics is also performed by antibiotic sensitivity test so that suitable antibiotic in semen extender can be used. The present study was, therefore, conducted to study the microbial load (bacteria & fungi), their biochemical properties antibiotic sensitivity of organisms present in the frozen semen of Holstein and Holstein crossbred cattle bulls.

Materials and Methods

A total of 30 randomly selected frozen semen doses from 30 cattle bulls comprising 7 Holstein Friesian and 23 Holstein crossbred cattle, were procured from one of the frozen semen banks in Gujarat. The semen was collected using sterilized artificial vagina adopting all aseptic procedures. Soon after collection the neat semen was evaluated by various parameters to ensure the quality of the semen before freezing. Once the quality is estimated to be good, the semen was diluted in egg yolk tris glycerol dilutor for filling, sealing and freezing in French medium straws (0.25 ml). The freezing by horizontal liquid nitrogen vapour freezing technique (9) at frozen semen bank. Mini French straws (0.25 ml) were collected for evaluation of microbial load using the standard plate count (SPC) method (10) by incubating at 37°C for 72 hr.

Mini French straws (0.25 ml) of frozen semen were collected for evaluation of microbial load using the standard plate count (SPC) method after incubating in nutrient agar plates, already checked for purity by incubating at 37 ^{0}C for 24 h before inoculating with the semen sample from 10^{-1} and 10^{-2} dilutions. Two SPCA plates were taken for a single batch of semen and 0.1 and 0.5 ml semen sample was inoculated in each plate. These plates were incubated at 37 ^{0}C for 24 and 48 h before examination for the final results. The bacterial colonies were counted with the help of a colony counter. The average colony count was calculated. The thawed semen was also examined for fungus, mucor and yeast by inoculating the samples in Sabaroud's dextrose agar at 37 ^{0}C for 24 h followed by subsequent examination of the organisms. Microorganism was also identified as Gram positive and Gram negatives. Antibiogram by using Ampicillin (A10) 10 mcg, Gentamycin (G10) 10 mcg, Erythromycin (E10) 10 mcg, Spectinomycin (Se100) 100 mcg were performed. Biochemical tests like methyl red test (11), Voges-Proskaur test (12), Citrate utilization test (13), Lead acetate paper strip test (14), Urea hydrolysis test (15), Nitrate reduction test (16), Indole test (17), Starch hydrolysis test (18) and Triple sugar iron test (19) were also performed to characterize the microorganism.

Result and Discussion

In present study average microbial load from frozen semen straws of 30 cattle bulls were assessed by standard plate count method using standard plate count agar and it is presented in table-1, from which eleven samples out of 30 were found positive for various bacterial isolates. The positive sample numbers are HF Crossbred-0588, HF Crossbred-0597, HF Crossbred-0445, HF Crossbred-0592, HF Crossbred-5003, HF Crossbred-0585, HF Crossbred-0573, HF-433, HF Crossbred-0598, HF Crossbred-0584, HF Crossbred-0591. These contain $0.2X10^2$ CFU/ml, $6.3X10^2$ CFU/ml, $1.6X10^2$ CFU/ml, $1.8X10^3$ CFU/ml, $0.2X10^2$ CFU/ml, $1.8X10^2$ CFU/ml, $0.9X10^2$ CFU/ml, $0.4X10^2$ CFU/ml, $0.8X10^2$ CFU/ml, $5X10^2$ CFU/ml, $1.1X10^{-3}$ CFU/ml respectively (Figure-1). Such frozen semen doses are acceptable to use for AI as per the Bureau of Indian Standard (BIS). Sample number HF Crossbred 0581 was found positive for fungal

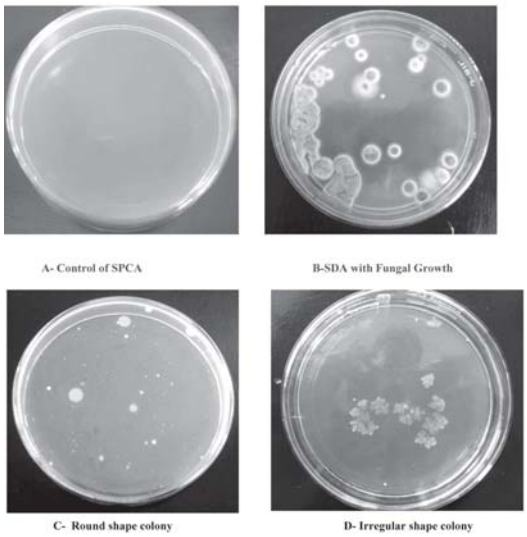

Fig. 1. SPCA with different types of colonies

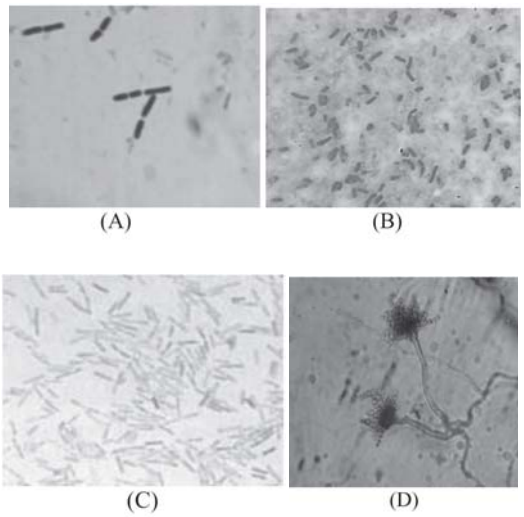

Fig. 2. (A)-Gram Positive Bacilli,
(B)-Gram Negative Coccobacilli,
(C)-Non acid-fast organism
(D)-Fungal Staining

growth when cultured on Sabouroud's dextrose agar followed by incubation in fungal incubator (Fig.1).

There were significant differences in the bacterial count between eleven cattle bulls. The morphological and cultural characteristics of isolated bacteria are summarized in Table-2. Bacterial isolate from bull number HF Crossbred-0588, HF Crossbred-0597, HF Crossbred-5003, HF Crossbred-0591 are Gram positive purple coloured *cocci* and they are also blue coloured non-acidfast and non-endospore formers. Bacterial isolate from bull number HF-433, HF Crossbred-0573 are Gram negative pinkish red coloured *coccobacilli* and they were also non-acidfast and non endospore formers**.** Bacterial isolate from HF-0445 is Gram positive purple coloured *bacilli* and they were also non-acidfast and non endospore formers**.** Bacterial isolate from HF Crossbred-0598 (A, B, C) were Gram positive purple coloured *bacilli* and they are also non-acidfast and among which only C was non endospore formers and A and B were endospore formers**.** Some semen sample of cattle bull like HF Crossbred-0592, HF Crossbred-0584 were producing two types of bacteria that is Gram positive *bacilli* and Gram negative *coccobacilli* and they are also non-acidfast and non endospore formers (Table 2 and Fig.2).

Bacterial contaminants in semen survive at -196°C in liquid nitrogen and had acquired a certain level of resistance to antibiotics (6). In the present study only 36.6% (11 out of 30) of frozen semen samples contaminated either with different bacteria or fungi that is lesser than earlier reports (7, 20) in crossbred cattle bulls showing 50% and 40% of frozen semen samples respectively. Seven different pathogenic bacteria from 100 frozen semen sample of cattle were also isolated and characterized (21). The level of bacterial contamination in the present study may be also due to non-aseptic condition during semen collection and processing or resistant to antibiotics used in semen extender/diluents. The bacterial contaminants of frozen semen have been classified as pathogenic, partially pathogenic and non- pathogenic.

Result of biochemical properties of bacterial isolates are summarized in Table.3 and Fig. 3, 4, 5, 6, 7 and 8. Each isolates are varying in their biochemical profile. HF-445(B) which was found

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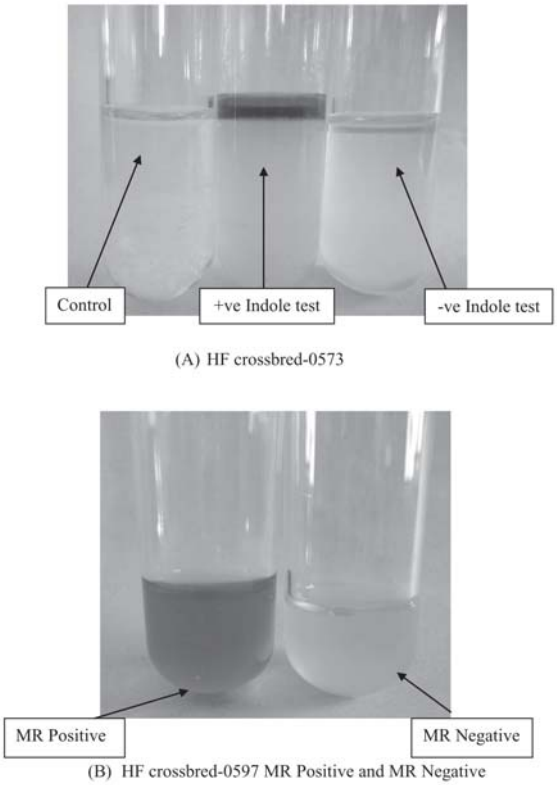


Fig.3. Biochemical tests: A) Indole Test & B) MR Test

positive for only starch hydrolysis test. So, it was difficult to identify each isolate in short span of time and limited biochemical tests. As per morphological, cultural and biochemical characteristics of isolates/colonies classified into A, B and C categories as elucidated in table No 2 & 3. As per the database available (22), the characteristics of isolates mentioned above (A, B & C) are similar to the *Micrococcus lutes, Streptococcus lactis, Alcaligenes faecalis, Staphylococcus aureus, Bacillus cereus, Proteus vulgaris, Shigella dysenteriae.* Some of isolates; *Micrococcus lutes, Proteus vulgaris* and *Staphylococcus aureus* are similar to finding of Abro *et al.,* (21) where they isolated 7 pathogenic bacteria from frozen semen of cattle and identified.

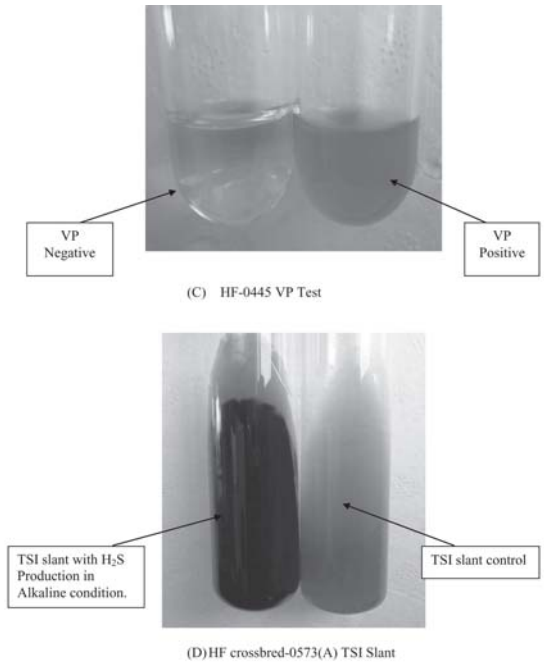


Fig. 4. Biochemical Test: (C) VP Test & (D) TSI slant

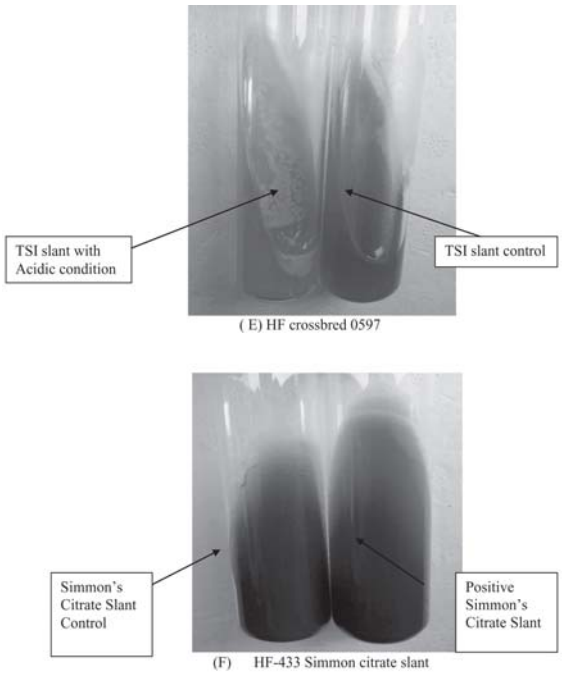
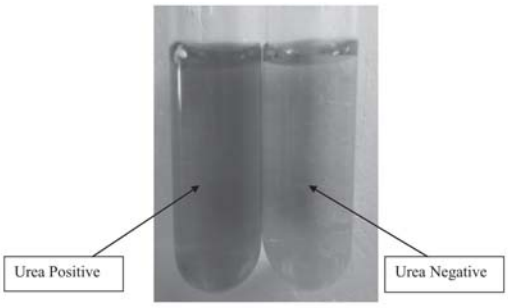
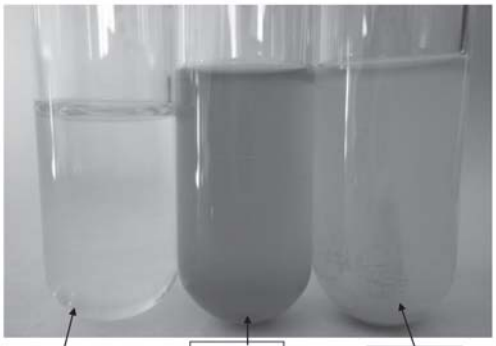


Fig. 5. Biochemical Test: (E) TSI Slant & (F) Simmon Citrate Slant

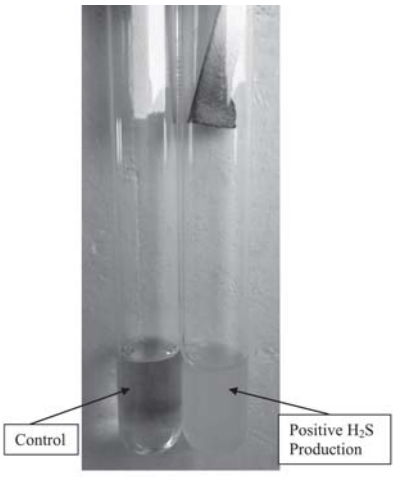


(G) HF crossbred-0573(a) Urea hydrolysis



(H) HF crossbred-0598 (B)

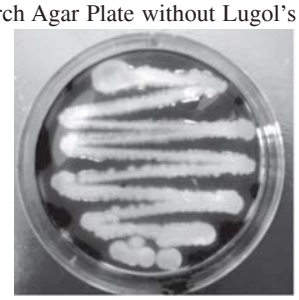
Fig. 6. Biochemical Test: (G) Urea Hydrolysis Test (H) Nitrate Reduction Test



(I) HF crossbred-0573(A) 2% peptone water

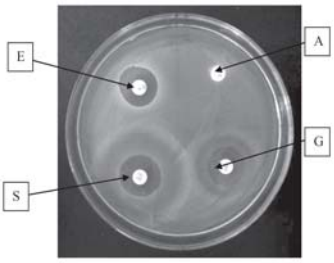
Fig. 7. Biochemical Test: (I) Lead Acetate Paper Test

Starch Agar Plate without Lugol's Iodine

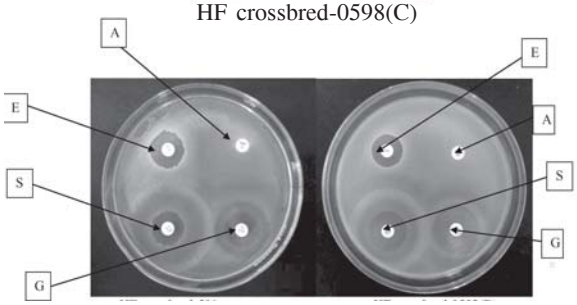


Starch Agar Plate with Lugol's Iodine

Fig. 8. Biochemical Test: (J) Starch Hydrolysis Test



HF crossbred-0598(C)



HF crossbred-591 HF crossbred-0592(B)

Fig. 9. Antibiotic Sensitivity Test A-Ampicillin, E-Erythromycin, S-Spectinomycin and G-Gentamycine

Table 1. Result of Standard Plate Count and Sabouraud's Dextrose Agar Plates

Sl.No. No.	*Breed/Bull No.*		*Dilution*	*Bacterial Growth (no. of colonies)*	*Average CFU/ml*	*Gram's reaction*	*Fungal Growth*
1	HF crossbred	0581	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
2	HF	0433	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
3	HF crossbred	0573	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
4	HF crossbred	0575	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
5	HF crossbred	0588	10^{-1} 10^{-2}	0.5ml - 20 No Growth	$0.2x10^2$	Gram +ve *cocci*	No Growth
6	HF crossbred	0580	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
7	HF crossbred	5004	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
8	HF	444	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
9	HF crossbred	0597	10^{-1} 10^{-2}	0.1ml - 5 0.5ml - 21 0.1ml - 1 0.5ml - 3	$6.3x10^2$	Gram +ve *cocci*	No Growth
10	HF crossbred	0584	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
11	HF	0445	10^{-1} 10^{-2}	0.1ml - 0 0.5ml - 6 No Growth 0.1ml -0 0.5ml - 1	$1.6x10^2$	Gram +ve *bacilli*	No Growth
12	HF crossbred	0592	10^{-1} 10^{-2}	0.1ml -14 0.5ml – 82 0.1ml -2 0.5ml - 11	$2.1x10^3$	Gram +ve *cocci* & *bacilli*	No Growth
13	HF crossbred	5003	10^{-1} 10^{-2}	0.1ml - 0 0.5ml - 1 0.1ml – 0 0.5ml - 0	$0.2x10^2$	Gram +ve *cocci*	No Growth
14	HF	0441	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
15	HF crossbred	0575	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
16	HF crossbred	0585	10^{-1} 10^{-2}	0.1ml - 2 0.5ml - 8 0.1ml - 0 0.5ml – 0	$1.8x10^2$	Gram +ve *cocci*	No Growth
17	HF crossbred	0580	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
18	HF crossbred	0584	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
19	HF crossbred	0581	$10^{-1}10^{-2}$	No Growth	-	-	ü
20	HF	0437	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
21	HF crossbred	0573	10^{-1} 10^{-2}	0.1ml - 1 0.5ml - 4 0.1ml - 0	$0.9x10^2$	Gram –ve *coccobacilli*	No Growth
22	HF	433	10^{-1} 10^{-2}	0.5ml - 0 0.1ml - 0 0.5ml - 2 0.1ml - 0 0.5ml – 0	$0.4x10^2$	Gram –ve *coccobacilli*	No Growth

23	HF crossbred	0575	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
24	HF	0432	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
25	HF crossbred	0577	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
26	HF crossbred	0585	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
27	HF Crossbred	0580	$10^{-1}10^{-2}$	No Growth	-	-	No Growth
28	HF crossbred	0598	10^{-1} 10^{-2}	0.1ml - 0 0.5ml – 4 0.1ml - 0 0.5ml - 0	$0.8x10^2$	Gram +ve *bacilli*	No Growth
29	HF crossbred	0584	10^{-1} 10^{-2}	0.1ml - 10 0.5ml – 2 0.1ml - 0 0.5ml - 0	$5x10^2$	Gram –ve *coccobacilli*	No Growth
30	HF crossbred	0591	10^{-1} 10^{-2}	0.1ml - 1 0.5ml – 6 0.1ml - 0 0.5ml - 0	$1.1x10^2$	Gram +ve *cocci*	No Growth

Table 2. Morphological and staining characteristics of bacterial species isolated from frozen semen of HF & HF crossbred bulls

Characters	**HF crossbred-0588**	**HF crossbred-0597**	**HF-445 (A)**	**HF-445 (B)**
Size	Small	Medium	Medium	Small
Shape	Round	Round	Round	Round
Margin	Entire	Circular	Circular	Circular
Elevation	Flat	Flat	Flat	Flat
Texture	Smooth	Rough	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque
Pigmentation	Yellow	-	-	-
Motility	Motile	Motile	Non –motile	Motile
Gram's staining	Gram Positive *cocci*	Gram Positive *cocci*	Gram Positive *cocci*	Gram Positive *bacilli*
Acid –fast	Non acid fast	Non acid –fast	Non acid-fast	Non acid-fast
Endo –spore	No endospore	No endospore	No endospore	No endospore

Characters	**HF crossbred -0592 (A)**	**HF crossbred-0592 (B)**	**HF crossbred -0592 (C)**	**HF crossbred -5003**
Size	Large	Large	Small	Small
Shape	Round	Irregular	Oval	Round
Margin	Circular	Irregular	Regular	Entire
Elevation	Slightly raised	Flat	Flat	Flat
Texture	Smooth	Rough	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque
Pigmentation	-	Golden	-	Yellow
Motility	Motile	Motile	Motile	Motile
Gram's staining	Gram Negative *cocco bacilli*	Gram Positive *bacilli*	Gram Positive *bacilli*	Gram Positive *cocci*
Acid –fast	Non acid-fast	Non acid-fast	Non acid-fast	Non acid fast
Endo –spore	No endospore	No endospore	No endospore	No endospore

Characters	HF crossbred-0585	HFcrossbred-0573 (A)	HFcrossbred-0573 (B)	HF-433
Size	Medium	Large	Large	Medium
Shape	Round	Round	Round with center concentrated	Round
Margin	Round	Entire	Entire	Entire
Elevation	Slightly raised	Flat	Flat	Submerged flat
Texture	Smooth	Smooth	Smooth	Rough
Opacity	Opaque	Opaque	Opaque	Opaque
Pigmentation	-	-	-	-
Motility	Motile	Motile	Motile	Motile
Gram's staining	Gram Positive *cocci*	Gram Negative *coccobacilli*	Gram Negative *coccobacilli* (very small)	Gram Negative *coccobacilli*
Acid –fast	Non acid-fast	Non acid-fast	Non acid-fast	Non acid-fast
Endo –spore	No endospore	No endospore	No endospore	No endospore

Characters	HF crossbred-0598 (A)	HF crossbred-0598 (B)	HF crossbred-0598 (C)	HF crossbred -0591
Size	Medium	Large	Medium	Large
Shape	Round	Round	Irregular	Round
Margin	Circular	Entire	Irregular	Entire
Elevation	Flat submerged	Flat	Submerged raised	Flat
Texture	Rough	Rough	Rough	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque
Pigmentation	-	-	-	-
Motility	Motile	Motile	Motile	Motile
Gram's staining	Gram Positive *bacilli*	Gram Positive *bacilli*	Gram Positive *bacilli*	Gram Positive *cocci*
Acid –fast	Non acid-fast	Non acid-fast	Non acid-fast	Non acid fast
Endo –spore	Endo spore formation	No endospore	Endo spore formation	No endospore

Characters	HF crossbred-0584 (A)	HF crossbred-0584 (B)	HF crossbred-0584 (C)
Size	Pinpoint	Medium	Medium
Shape	Small	Irregular	Round
Margin	Round	Irregular	Entire
Elevation	Slighty raised	Submerged raised	Flat
Texture	Smooth	Rough	Smooth
Opacity	Opaque	Opaque	Opaque
Pigmentation	-	-	-
Motility	Motile	Motile	Motile
Gram's staining	Gram Negative *coccobacilli*	Gram Positive *bacilli*	Gram Positive *cocci*
Acid –fast	Non acid-fast	Non acid-fast	Non acid fast
Endo –spore	No endospore	No endospore	No endospore

Table 3. Biochemical properties of isolated bacterial species

Sample/bull No.	I	MR	VP	CUT	2% Peptone	UH	NRT	TSI	SH
HF crossbred-0588	-	-	-	+	-	-	-	S.-Alkaline	
								B.- Alkaline H.G.	-
HF crossbred -0597	-	+	-	-	-	-	-	S.-Acidic	
								B.- AcidicH.G.	-
HF-445 (A)	-	+	-	-	-	-	-	S.-Acidic B.H. G.	-
HF-445 (B)	-	-	-	-	-	-	-	-	+
HF crossbred-0592 (A)	-	-	-	-	-	-	+	S.- Alkaline	
								B.- AlkalineH.G.	-
HF crossbred-0592 (B)	-	+	-	-	-	-	+	S.- Alkaline	
								B.- AcidicH.G.	+
HF crossbred-0592 (C)	-	-	-	-	-	-	+	S.- Alkaline	
								B.- Acidic H.G.	+
HF crossbred-5003	-	-	-	-	-	-	-	-	+
HF crossbred-0585	-	+	-	-	-	+	-	S.-AcidicB.H. G.	+
HF crossbred-0573 (A)	+	+	-	-	+	+	-	S.- Alkaline	
								B.-BlackH_2S Prroduce G.	-
HF crossbred-0573 (B)	+	+	-	-	+	-	-	S.-Acidic	
								B.- AcidicH.G.	+
HF-433	+	+	-	-	+	-	-	S.-Acidic	
								B.- AcidicH.G.	+
HF crossbred-0598 (A)	+	-	+	-	+	-	-	S.- Alkaline	
								B.- AcidicH.G.	+
HF crossbred-0598 (B)	-	-	-	-	-	-	+	S.- Alkaline	
								B.- AcidicH.G.	+
HF crossbred-0598 (C)	-	+	-	-	-	-	-	S.-Acidic	
								B.- AcidicH.G.	-
HF crossbred-0584 (A)	-	+	-	-	-	-	+	S.- Alkaline	
								B.- AcidicH.G.	-
HF crossbred-0584 (B)	-	+	-	-	-	-	+	S.-Acidic	
								B.- AcidicH.G.	+
HF crossbred-0584 (C)	-	+	+	+	-	-	-	S.- Alkaline	
								B.- AcidicH.G.	+
HF crossbred-0591	-	+	+	-	+	-	-	S.- Alkaline	
								B.- AcidicH.G.	+

I : Indole Production Test, MR: Methyl Red Test, VP : Voges Proskauer's Test, CUT : Citrate Utilization Test, 2% Peptone: Lead Acetate Paper Strip Test, UH : Urea Hydrolysis Test, NRT : Nitrate Reduction Test, TSI: Triple Sugar Iron Test, S. –Slant, B. – Butt, H. – H_2S production, G.- Gas Production, SH : Starch Hydrolysis Test

Table 4. Result of Antibiotic Sensitivity test (zone of inhibition in mm)

Sample/bull No.	Zone of Inhibition			
	Ampicillin	Erythromycin	Gentamycin	Spectinomycin
HFcrossbred-0588	No Zone	30	26	30
HFcrossbred-0597	No Zone	17	21	20
HF-445 (A)	No Zone	29	19	19
HF-445 (B)	No Zone	20	20	19
HFcrossbred-0592 (A)	No Zone	No Zone	10	No Zone
HFcrossbred-0592 (B)	No Zone	17	25	20
HFcrossbred-0592 (C)	No Zone	17	20	20
HFcrosbed-5003	No Zone	30	26	30
HFcrossbred-0585	No Zone	No Zone	No Zone	5
HFcrossbred-0573 (A)	No Zone	No Zone	20	14
HFcrossberd-0573 (B)	No Zone	17	15	22
HF-433	No Zone	10	15	28
HFcrossbred-0598 (A)	No Zone	17	18	20
HFcrossbred-0598 (B)	No Zone	27	20	20
HFcrossbred-0598 (C)	No Zone	17	16	28
HFcrossbred-0584 (A)	No Zone	25	18	24
HFcrossbred-0584 (B)	No Zone	17	16	28
HFcrossbred-0584 (C)	No Zone	17	16	28
HFcrossbred-0591	No Zone	18	17	17

The results of antibiotic sensitivity pattern in bacterial isolates shown in Table-4 and Figure-9, which was done by agar disc method. All bacterial isolates exhibited variable pattern against Ampicillin, Erythromycin, Gentamycin and Spectinomycin. The concentrations of antibiotics were 10µg, 10µg, 10µg and 100 µg respectively. Among all these antibiotics all bacterial isolates are resistance against Ampicillin.

Bacterial contamination in frozen semen first leads to the production of macrophages and polymorphonuclear granulocytes that is first line of defense against bacteria. Both the cells generate reactive oxygen species that in turn impair sperm function and reduces its fertilization capability (4, 23). Bacteria also adhere to spermatozoa and interfere with their motility (1, 24, 25). Microbes can also have direct reaction with acrosome or indirectly reacts by producing toxins (23, 26).

It is documented that bacteria in the semen is controlled by using antibiotics in freezing diluents. Conventially benzyl penicillin and spreptomycin sulphate alone or in combination is added at a concentration of a 1000µg/ml respectively (27). In the present study we investigated 4 antibiotics (Ampicillin, Erythromycin, Spectinomycin, Gentamycine) on all that nineteen bacterial isolates. Except Ampicillin, all antibiotics inhibited growth of all

bacterial isolates but their deleterious or toxic effect on spermatozoa to be evaluated before using them in cryopreservation of cattle semen. Thus it can be concluded from the present study that bacterial load was estimated below the standard counts but increasing bacterial load in the semen leads to detoriation of semen quality in terms of motility and viability and also causes morphological alterations in the sperm cells which are manifested in terms of increased sperm abnormalities.

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Reliability in Transformation of the Basidiomycete *Coprinopsis cinerea*

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Abstract

Transformation of the basidiomycete *Coprinopsis cinerea* makes use of unicellular haploid asexual spores called oidia. Protoplasts of oidia are generated by a cellulase/chitinase enzyme mix. Protoplasts and DNAs are incubated together in 25 mM Ca^{2+} and 5% PEG (polyethylene glycol) 4000 on ice and more PEG is added (23% final concentration) after a 'heat shock' step at RT (room temperature). Upon regeneration on selective media, transformation rates of several hundreds of clones might be obtained per 1 µg DNA and 10^7 protoplasts. Although the technique has been invented 25 years ago by Binninger *et al.* (1), there are reoccurring pitfalls in the method that can cause failure. Successful transformation needs a good amount of skilful knowhow about the fungus and the method. Here we present our experiences with *C. cinerea* transformations, call attention to potential flaws and to optimal handlings in fungal cultivation, harvesting, protoplasting, transformation, and subsequent regeneration of the fungus.

Keywords: Transformation, protoplasts, *C. cinerea* vectors, oidia, basidiomycete

Introduction

Transformation in *Coprinopsis cinerea* has first been described by Binninger *et al.* in 1987 (1). *C. cinerea* produces unicellular aerial spores (oidia) with one haploid nucleus [(2,3); Fig. 1] and Binninger *et al.* (1,4) used these to generate protoplasts for Ca^{2+}, in-ice-incubation-, heat shock-, PEG-mediated vector transformation. Around 100 transformants per experiment (about 33 per µg vector DNA and per 10^7 total and 10^6

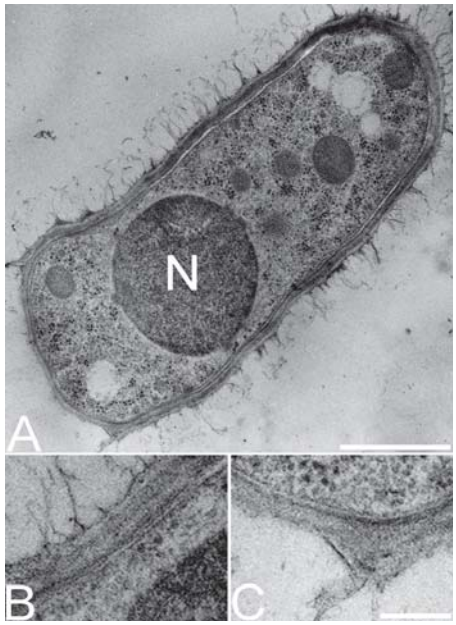

Fig. 1. An unicellular oidium of *C. cinerea* strain AmutBmut with a haploid nucleus (N), a bilayered outer cell wall with hair-like structures contributing to a gelatinous layer surrounding the spore, and a single-layered ruptured septum cell wall found at the side of former attachment to another spore (2,3). A. Transmission electronic photograph of the complete spore (size bar = 1 µm), and enlarged views B. of the outer cell wall of the oidium and C. of the septal cell wall (size bar = 0.2 µm). Figure modified from (3).

viable protoplasts) were obtained by these authors (1,4), rates comparable to those of protoplasts made from fungal mycelium (1,5,6). Similar high rates in oidia transformation were reported in studies by other groups (7-13). Circular or linearized DNA or also single-stranded DNA can be used for the transformation of *C. cinerea* (4,10) and in all cases ectopic integration of the foreign DNA into the host chromosomes takes place (1,6-12). Importantly, two or more plasmids may be transformed at the same time into a same protoplast allowing through complementation of auxotrophies or through conferring antibiotic resistances indirect selection for transformed genes having no easily selectable phenotypes and, in the following, studies of different genes at the same time (7-9,11,13-16).

Many a time in *C. cinerea* laboratories, transformations are however also unsuccessful. Such failures are not published but negative experiences are told among the international *Coprinopsis* community. In over 20 years of research with *C. cinerea* transformation by the senior author, five longer periods of failure in transformation were experienced by members of the own research teams, distributed over three different labs in three different countries (Oxford, UK; Zurich, Switzerland; Göttingen, Germany). Accordingly, *C. cinerea* transformation has among some researchers a reputation to be a difficult technique. Commonly in situations of longer failure, the water used was made responsible or the enzyme used, or strains for transformation were suspected to have changed properties or the DNAs being of not good enough quality. Ultra-pure water had been bought and new enzyme batches, transformation strains newly acquired from other labs or replaced by other ones, and DNAs isolated by different methods. These measures not necessarily helped. Observations suggest that a personal factor has an important position in these negative results. Changing a person in charge of transformation to a fresh investigator rendered the outcome positive, also with the same water, enzymes, strains and DNAs. New laboratories were established at two occasions and new people were kept unladen from the idea that transformation can be difficult. Persons so introduced to transformation were excellent in performance, with no complaints and no experiences of technical difficulties. Periods of about 5 years were without major troubles. However, some newcomers to *C. cinerea* transformation were subsequently unable to perform the technique. In some instances, wrongly made up buffers, media and solutions were proven to be the cause and this could quickly be solved. In other instances, persons failed by changing details in the method ('for optimizing the protocol') and wanting to adapt to what they felt to be better in handling or to be more accurate in procedure. Otherwise well practiced researchers so never made transformation work, also not when keeping to the established procedure. The resulting idea of *C. cinerea* transformation being a 'bad technique that needs to be reformulated' turned out to be infectious. Lab colleagues could then also not transform *C. cinerea* or when, only with very low success of obtaining only few transformants. Consequently, there was need for the senior author to show in own action that the method perfectly works, even after having self not been active for times in the laboratory. Upon such demonstration, the transformation technique kept on going until some chance of personnel. A number of times, the *C. cinerea* transformation procedure had thus been taught from scratch (U. Kües, pers. observations).

With this paper, we intend to describe and explain the detailed steps and actions in the *C. cinerea* transformation procedure, to call attention to sensitive phases in handling, and to point out potential pitfalls that can cause failure.

Material and Methods

Coprinopsis cinerea *strains and plasmids:* Monokaryotic *C. cinerea* strains FA2222 (*A5*, *B6*, *acu1*, *trp1.1,1.6*) and PG78 (*A6*, *B42*, *pab1*, *trp1.1,1.6*) (16) were used in this study and

homokaryotic strains EN117.9 (*A6m*, *B5*, *ade8*) (17) and the UV-mutant 7K17 of strain AmutBmut (*A43mut*, *B43mut*, *pab1*) having mutations in the mating type loci (10,16; Granado *et al.* unpublished). The following plasmids were used: the pUC9 derivative pCc1001 with the *C. cinerea* wild type *trp1* gene (1), the pUC13 derivative pST17 (18,19) and the pTZ18R derivative pPAB1-2 (10) with the *C. cinerea* wild type *pab1* gene, and cosmid pCRS1 from the *C. cinerea* JV6 Lorist 2 cosmid library with the *ade8* wild type gene (18,20). pYSK7 as a derivative of the yeast-shuttle vector pRS426 with the *C. cinerea* wild type genes *pab1* and *lcc1* (13) and construct pYPH3 (Hoegger *et al.* unpublished) with gene *lcc1* in pYSK7 replaced by a *Pleurotus sapidus* putative versatile peroxidase gene (GenBank accession number AM039632) were used in cotransformation of strain FA2222 together with pCc1001.

DNA preparation: Plasmid isolation from *Escherichia coli* performed in mini-prep form is a modification of the method of Birnboim and Doly (21). *E. coli* strains with the respective plasmids are cultivated overnight (ca. 14 h) at 37°C in 3 ml LB-medium (22) supplemented with the appropriate antibiotics on a shaker at 180 rpm. Cells are harvested in two portions in a 1.5 ml Eppendorf tube by centrifugation (1 min; 16.000 x *g*). The resulting cell pellet is resuspended in 75 µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). 150 µl alkaline lysis solution (always freshly prepared by mixing 1:1 0.4 M NaOH and 2% SDS) is added and mixed with the cell suspension. After incubation for up to 20-30 min at RT (room temperature; the solution needs to become viscous and clear), 500 µl of renaturation solution (always freshly made by mixing in 1:3:6 portions 5 M NaCl, non-autoclaved 3 M Na acetate of pH 4.8 and sterile H_2O) is added and mixed (white flocks form from cell debris). The mixture is kept at -20°C for 10-20 min to improve precipitation of unwanted cell debris. Afterwards, the cell debris together with the chromosomal DNA is removed by centrifugation (20 min; 16.000 x *g*). The supernatant with the plasmid DNA is poured into a new 1.5 ml tube and the DNA is precipitated by addition of 750 µl isopropanol, mixing the solutions at RT (multiple small gas bubbles will form to sparkle to the surface) and centrifugation (15-20 min; 16.000 x *g*; the junction between tube and cap should be turned to the outside of the rotor in order to mark the side of DNA pelleting during centrifugation). The supernatant is poured off and the remaining liquid sucked off by turning the opened tube upside down onto clean towelling paper. The resulting pellet is then washed by rinsing the tube with 500 µl 70% ethanol and subsequent short centrifugation (5 min; 16.000 x *g*). The supernatant is poured off, the remaining liquid sucked off by turning the opened tube upside down onto clean towelling paper, and the visible pearly pellet of DNA and RNA at the bottom of the tube air-dried at RT. Note that more clean DNA might be distributed as a transparent film over the wall of the tube at the side of the junction with the cap (depends on the brand of tubes used). DNA and RNA are resuspended in 50 µl sterile H_2O under repeatedly rinsing the wall of the tube with the liquid (until the liquid easily runs in droplets down). The DNA concentration is determined by agarose gel electrophoresis (22).

In addition, purified maxi-prep DNA of cosmid pCRS1 for this study was prepared by CsCl-ethidium bromide gradient centrifugation (22) and purified pST17 DNA by a Qiagen Small Scale Plasmid Purification Kit (Qiagen GmbH, Hilden, Germany).

Fungal transformation as modified by Granado et al. (10): *C. cinerea* strains are grown at 37°C on fresh YMG or YMG/T (*trp* auxotrophs) agar medium until the whole plate is covered by mycelium. Plates are either inoculated by a small piece of agar with mycelium from freshly grown precultures placed in the centre of the plate or by four mycelial pieces placed onto the agar at equal distances to each other and about 2-2.5 cm apart from the edges of the Petri dish (9 cm in Ø) (Fig. 2). For optimal growth, plates are transferred into aerated dark boxes whose bottoms are covered by wet towelling paper to

keep a high humidity in the air. Petri dishes with cultures for transformations should not be set directly on the towels but onto a spacer (e.g. another Petri dish) in between to avoid any contaminations on the outside of the plates by transfer from the wet tissues. Boxes with plates should not be set directly onto the warm bottom of an incubator to prevent heat accumulation in the box. Monokaryons of *C. cinerea* can be used as such but for transformation of strains with defects in the *A* mating type genes, fully grown plates should be transferred either for 2 days at 37°C or for 4 days at 25°C and high humidity into white light (light intensity 20-25 µE m^{-2} s^{-1}; light source e.g. Osram L40/25; Osram AG, Munich, Germany) for induction of oidia production (10,6,23).

For harvest of spores, ca. 10-15 ml sterile H_2O are poured (from a bottle with sterile water) onto the aerial mycelium of a fully grown plate. Spores are released from the aerial mycelium by scraping with a sterile blunt spatula. The edge of the dry plastic Petri dish is sterilized by brief flaming with a Bunsen burner and the spore suspension is directly poured over the sterilized edge of the plate into a sterile thistle funnel (3-4 cm in Ø), which contains a layer of glass wool (Fig. 3A,B). The spores are filtered through the glass wool into a sterile test tube, thereby leaving agar pieces and mycelial debris behind. The filtered spore solution is then transferred into a 30 ml Sterilin polystyrene tube (item code 128A, Sterilin Ltd, Newport, UK; Fig. 3C,D) and centrifuged (5 min; 2.600 x *g*) against another Sterilin tube filled up from a spray bottle with water to a same volume as the spore suspension. The pelleted spores are resuspended in 5 ml freshly prepared sterile MM buffer and centrifuged (5 min; 2.600 x *g*). The supernatant is discarded and the enzymatic digest of the fungal cell walls started with resuspension of the spore pellet in 2 ml osmotically stabilized cellulase/chitinase solution. The mixture is incubated at 37°C with the caps of the tubes loosely closed to allow aeration as well as protection. The tubes are either gently shaken (110 rpm) in slightly tilted

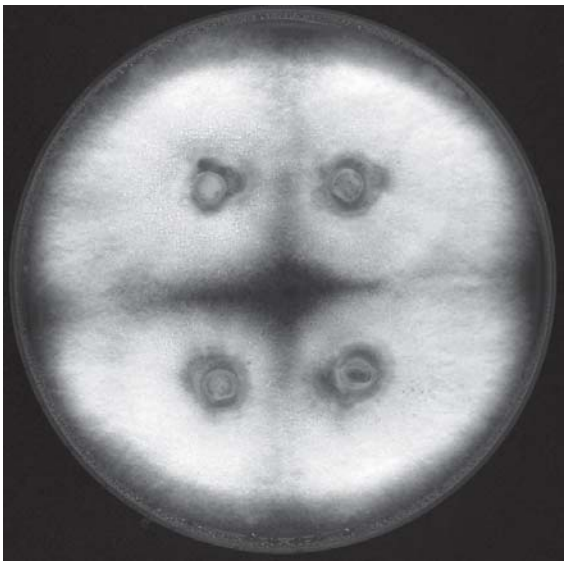

Fig. 2. YMG/T agar plate inoculated with four mycelial agar pieces of *C. cinerea* FA2222 after six days growth at 37°C in the dark.

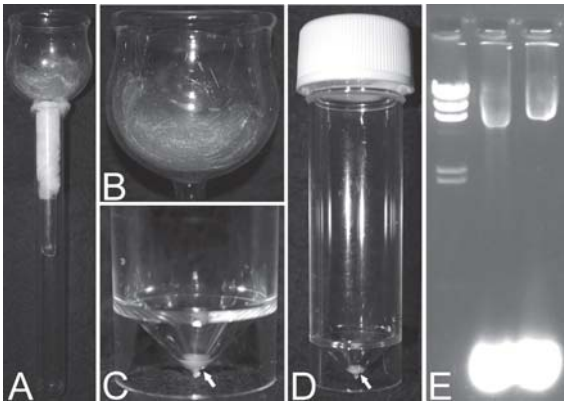

Fig. 3. Selected materials required for transformation. A. and B. Thistle funnel with glass wool: the funnel is fixed in the test tube with a layer of cotton wool and for autoclaving the opening of the funnel and the cotton wool should be covered with aluminium foil. C. and D. Sterilin 30 ml tube after harvest of oidia from strain FA2222 grown for 8 days at 37°C: the arrows point to the pellet of spores. E. Ethidium bromide-stained agarose gel with 200 ng marker λ-DNA-*Hin*dIII (lane 1) and 1 µl pCc1001 mini-prep DNA (lane 2) and 1 µl pYSK7 mini-prep DNA (lane 3) with RNA seen as a large spot at the bottom of the gel.

position (30° angle) or laid raked down onto a stand to produce an as large surface as possible without coming too close to the edge of the tube for spilling solution. After 3-4 h incubation, a first sample is taken (ca. 3 µl) and the grade of protoplast formation is determined by examination under the microscope. The level of protoplasting defines whether samples are further incubated or whether further enzyme mix (in 1 or 2 ml portion) might be added. At a good level of protoplasting (usually ca. 50-70%), the enzymatic digest is stopped by addition of 5 ml sterile MMC buffer and gentle centrifugation (10 min; 640 x *g*). The supernatant is slowly poured off with caution that cells and protoplasts are minimally dispersed and that the slippery pellet does not get lost. The pellet is washed another time by 5 ml MMC buffer, with centrifugation (10 min; 640 x *g*) and carefully discharging the supernatant. The pellet is carefully resuspended in 300-500 µl MMC buffer to densities of about 0.2-2 x 10^8 cells/ml, depending on the strain, the quality of protoplasting and eventual losses of cells during the handling in the protoplasting procedure. Aliquots of each 50 µl are transferred to sterile 1.5 ml tubes. For transformation, plasmid DNAs (usually 1 µg per plasmid in single transformation or each 1 µg per different plasmid in cotransformation) and 12.5 µl PEG/$CaCl_2$ solution are added and gently mixed with the protoplasts. The tube(s) are placed for 20 min on ice. Then, 500 µl PEG/$CaCl_2$ solution are added to the protoplasts and gently mixed to further incubate for 5 min at RT ('heat shock treatment'). Finally, 1 ml of sterile STC buffer is added and mixed. The transformation mix is spread in usually four portions (ca. 390 µl per plate) onto freshly prepared solid regeneration medium (if required with appropriate supplements). The plates are incubated at 37°C in aerated dark boxes on wet towelling paper. After ca. 3 to 4 days, first transformants might arise and hyphal growth should be monitored using a binocular. When the first mycelium arises, remaining free liquid should be removed from the surface of the plates by drying them shortly under a laminar flow cabinet. Growing colonies should be picked by a sterile tungsten needle and transferred onto fresh medium under appropriate selection (e.g. minimal medium for growth of prototrophs). Care must be taken that all mycelium of grown transformants is harvested from the plates prior to their further incubation at 37°C for growth of additional transformants. Every day, plates should be checked for harvest of new transformants. Usually, further transformants will appear for the next 4 to 6 following days. If too many transformants appear at a time or if transformants grow too fast, the process might be slowed down by incubating the plates at lower temperature (RT).

Media (1,10,24,25): YMG or YMG/T medium (per l: autoclave separately 4 g yeast extract, 10 g malt extract, and for YMG/T only 100 mg tryptophan in 900 ml bidest. H_2O and 4 g glucose in 100 ml bidest. H_2O to mix the two solutions after autoclaving; if required add 1% agar to the first solution for solidification); regeneration medium (per l final volume: autoclave separately 25 ml stock solution A, 1 ml stock solution B, 10 ml stock solution C, optional 50 mg adenine sulphate, 2 g L-asparagine, 172 g sucrose, 5 g soluble starch, filled up with bidest. H_2O to exactly 900 ml to then add 1.2% or 1% agar and 5 g glucose filled up with bidest. H_2O to exactly 100 ml to mix the two solutions after autoclaving; if required add 100 mg of a respective amino acid per l, and 5 mg *para*-amino benzoic acid per l); minimal medium (as regeneration medium but without the optional 50 mg adenine sulphate, 172 g sucrose, and 5 g soluble starch and with 10 g instead of 5 g glucose per l; if required add 100 mg of a respective amino acid and 5 mg *para*-amino benzoic acid per l); stock solution A (per l final volume: 40 g KH_2PO_4, 90 g Na_2HPO_4, 11.6 g Na_2SO_4, and 20 g di-ammonium tartrate, fill up to 1 l with bidest. H_2O; store in fridge over some droplets of chloroform); stock solution B (per l: 40 mg thiamine in bidest. H_2O; autoclave and store in fridge); stock solution C (per l: 25 g $MgSO_4$ x 7 H_2O in bidest. H_2O; store in fridge over some droplets of chloroform). Agar

concentrations in the media refer here to Serva agar (cat. No. 11396, Serva Electrophoresis GmbH, Heidelberg, Germany) and its specific gelling properties.

Solutions (1,10,23): MM buffer [always prepare freshly in 2:1:1 portions from sterile stocks of 1 M mannitol, 0.2 M Na maleate (pH 5.5), and bidest. H_20]; MMC buffer [always prepare freshly in 2:1:0.1:0.9 portions from sterile stocks of 1 M mannitol, 0.2 M Na maleate (pH 5.5), 1 M $CaCl_2$, and bidest. H_20]; cellulase/chitinase solution [per 20 ml MM buffer: 800 mg cellulase "Onozuka" R-10 from *Trichoderma viride* (1 U/mg, cat. No. 16419, Serva Electrophoresis GmbH, Heidelberg, Germany); 20 mg (standard) chitinase from *Streptomyces griseus* (product number C6137 with guaranteed $\geq$ 200 units/g, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany); filter sterilize and store in 2 ml aliquots at -20°C]; PEG/$CaCl_2$ [10 g PEG (polyethylene glycol) 4000 (product number 807490, Merck KGaA, Darmstadt, Germany), 400 µl 1 M Tris-HCl (pH 7.5), and 1 ml 1 M $CaCl_2$, fill up to 40 ml with bidest. H_2O; filter sterilize and store in 5-10 ml portions at -20°C]; STC buffer (1 M sorbitol, 25 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5); autoclave). Note that the stock solutions for MM and MMC buffers as well as the STC buffer and any bottle with bidest. H_20 might be autoclaved again after usage to avoid that any accidental contamination will grow in these in between two experiments.

Results and Discussion

DNA for transformation: The quality of DNA is very important for transformation. We use in transformation plasmid DNA prepared by the mini-prep method modified after Birnboim and Doly (21) as described in the materials and methods. Such prepared DNA contains also large amounts of RNA (Fig. 3E). RNA in DNA samples helps to precipitate the DNA (26). However, this RNA can be of further benefit for the success of transformation as indicated by comparative transformation experiments. Transformation of strain EN117.9 for example with 1 µg pCRS1 isolated by the mini-prep method resulted in 171 *ade8*$^+$ transformants whereas no transformant was obtained with 1 µg of pCRS1 purified by CsCl-ethidium bromide gradient centrifugation from RNA. Similarly in two different transformation experiments of strain PG78, 91, respectively 164 different *pab1*$^+$ transformants were obtained with 1 µg pST17 prepared by the mini-prep method and only one, respectively 11 *pab1*$^+$ transformants with 1 µg pST17 prepared by a Qiagen Plasmid Purification Kit including the usual RNAse treatment. Costa *et al.* (15) reported for the same strain similar low transformation rates from cotransformation experiments: 61 transformants were obtained from three experiments with 2 µg pST17 and 5 µg of a plasmid pSUPER-GFP of Qiagen-Kit-purified DNA.

Notably, in other experiments with PG78 protoplasts and pST17-RNA-mixtures from mini-preps we reached transformation rates of 300-700 clones/µg vector DNA, and with pCc1001-RNA-mixtures and pPAB1-2-RNA-mixtures transformation rates of each up to 300 clones/µg vector DNA. Similar transformation rates of up to 450 clones/experiment for strain PG78 and plasmid pPAB1-2 were reported by Granado *et al.* (10) who, although not mentioned at the time in the paper, also used mini-prep DNA. Single-stranded nucleic acids (either RNA or single-stranded DNA) applied as carrier RNA or carrier DNA in transformation of other fungi including the basidiomycete *Pleurotus ostreatus* has been shown to well enhance transformation frequencies (27-32). The mechanism(s) by which carrier RNA and DNA increase transformation rates remain(s) elusive. These extra nucleic acids may protect the plasmid DNA against nucleases by distracting them from the plasmids (27), which might happen outside of the cells or after transfer in the cells. Other papers on fungal transformation speculate on a cell-wall-binding function that helps either to cover all available DNA binding sites so that the plasmid DNA remains in solution for uptake into the cells (29,30) or to make the cell wall loose and better porous for transfer of plasmid DNA (33). If so,

this might affect transformation with spheroplasts.

An important factor is surely the overall purity of the DNA. DNA prepared by a traditional mini-prep method might be considered as only coarsely purified. Protein and other cell debris might not be well separated, especially when using a renaturation buffer made up of either only K acetate or only Na acetate (22). However, if 0.5 M NaCl end-concentration is added to the renaturation buffer, if the volume of renaturation buffer (500 µl) is large enough to allow easy mixing with the slimy-viscous sample with the denaturated DNA, protein and other cell debris, and if in addition the mixture is cooled down at -20°C (up to freezing to ice), protein and cell debris well precipitate in granular flocks that form to a compact pellet upon centrifugation. Pouring the supernatant after centrifugation into a new tube ensures that all cell debris are left behind and no attempts should be done to rescue also the small volume of liquid remaining in the tube (such as by pipetting). In the next step after isopropanol precipitation of DNA and RNA (done at RT to not favour precipitation of any left other substance), centrifugation, pouring off the supernatant, and sucking off all remaining liquid by towelling paper will remove any excessive NaCl and any other soluble compound possibly left. Repeating this step upon the 70% ethanol wash step will further contribute to obtain highest DNA (with RNA) purity.

***Growth of* C. cinerea:** *C. cinerea* strains differ in speed of growth and in number of oidia produced in the aerial mycelium (16,34). Strain FA2222 for example takes 10-12 days and strain PG78 6-7 days at 37°C to fully cover with mycelium the surface of YMG/T medium in a 9 cm Ø Petri dish upon inoculation with a single mycelial piece in the middle of the plate. The freshly grown mycelium needs about 12-24 h to produce aerial spores (2,35), why a fully grown plate might be better stored another 1 or 2 days at 37°C prior to use which will increase absolute spore numbers by 2.5 fold. Two days after a plate is fully grown, strain FA2222 gives rise to 5 x 10^9 oidia/plate and strain PG78 8 x 10^8 oidia/plate (16). Oidia are only short lived and may lose germination ability already after two further days under temperature stress. A long period needed for a strain to fully grow over a plate can thus result in high portions of non-germinable oidia (36). Survival rates will be better with shorter incubation rates at 37°C. This can be achieved by placing four pieces of mycelium onto a YMG/T agar plate for cultivation (Fig. 2). Cultivation times for mycelium of strain FA2222 to fully cover the agar are thus reduced to 7-8 days and for strain PG78 to 4-5 days.

Stress for the spores should be best avoided. High water activity (a_w value) favoured by mycelium and spores is ensured by using fresh agar plates for fungal growth with only 1% agar (if employing as we agar from Serva; note that agars from different suppliers might have different gelling properties that can differentially influence the a_w value of the medium). Keeping the humidity in the air high by incubating plates in dark boxes on wet tissues helps further the well-being of mycelium and spores.

Best is to use the freshly grown plates directly for transformation. To have always fresh plates at hand for transformation can be ensured by regularly inoculating plates on a daily basis. If transformation does not need to be as perfect (in high numbers of transformants), it is possible to use plates that after completing growth at 37°C were stored at lower temperature, so at RT for 1 or 2 days or at 4°C for several days (10). For own comfort, plates ready for use should be available on Thursdays and Fridays as transformation is best done on these days to then start picking first regenerated clones on Monday or Tuesday.

Harvest of spores: Important during harvests of spores is to avoid any contamination. Plates should be dry from the outside. During growth, this can be warranted by not placing plates directly onto the wet tissues in the dark box.

Further to avoid contamination, handling in harvest should be fast (but not hectic). An aliquot

of sterile water can quickly be poured onto the mycelium from a flask so that the hydrophobic aerial mycelium is partially covered (needs about 10-15 ml). Scraping quickly the mycelium with the sterile blunt spatula (a sharp edge will injure the agar) distributes the liquid over the whole surface and brings spores and mycelial debris into solution. Prior to pouring off the spore solution into a funnel, short flaming the outer plastic helps to kill any attached microbes that possibly could contaminate the spores. The bell-shaped thistle funnel (Fig. 3A,B) used for filtration ensures that the glass wool nestles well to the glass surface of the funnel´s bottom and that also during spore suspension filtering, unlike in the case of a conoid funnel where pouring off the oidia suspension can easily displace the glass wool. When pouring off the spore solution, no attempt should be made to harvest the last droplet of spores from the plate as this extra handling might increase the risk of unwanted contamination and the spores lost in this left-over will not majorly change the absolute number of spores harvested.

The filtered spore suspension is sterile transferred into a Sterilin 30 ml tube (Fig. 3C,D). This translucent tube has several advantages also for the following protoplasting procedure. The conical form of the bottom allows compact pelleting of spores and protoplasts and the sizes, colours and behaviour of pellets can easily be observed through the plastic and, with experience, spore and protoplast amounts be judged from pellet size (Fig. 3C). A pellet size as shown in Fig. 3C (with ca. 4 mm height along the slant of the tube) corresponds to about 10^8 spores that might last for 4 to 8 parallel transformation samples. Fewer spores should not be taken for experiments since pelleting works less well for lower amounts of spores and protoplasts and there is always some loss of cells. If more spores are needed for more transformations to be done in parallel, oidia from two (or more) plates might be combined. It is however also not advisable to harvest too many spores in one tube. Above 10^{10} cells, relations between cell numbers and the volume and total activity of the enzyme mix might become negative, for example also with regards to good aeration.

After protoplasting, in best case the pellet is only slightly reduced in size and the colour will have changed from compact whitish of the undigested spores to gleaming white of the protoplasts. Furthermore with Sterilin tubes, when slowly pouring off supernatants after centrifugation, it is easy to keep an eye on the pellets so that they not accidently slip away, out of the tube. Important is also the behaviour of the protoplasts with the polysterene walls of the tubes. Protoplasts are charged and upon gentle centrifugation (10 min; 640 x *g*) attach comparably well as a loose pellet to the walls. Centrifugation in other plastic tubes (such as centrifuge tubes made of polypropylene) might not be much a problem with the undigested spores but the slippery protoplast pellets will repel from the plastic with a great danger of loss during handling.

Protoplasting: After harvesting and washing spores with sterile MM buffer, an enzyme mix of cellulase “Onuzuka” R-10 and Sigma C6137 chitinase in MM buffer as osmotic stabilizer is added to digest the spore cell walls. Oidia of *C. cinerea* have bilayered hyphal cell walls and single-layered septal cell walls and are surrounded by a gelatinous mucilage [(2,37); Fig. 1]. From other cells of the fungus (38-41) and from cell wall analyses of the basidiomycete *Schizophyllum commune* (42), it is expected that the cell walls contain chitin and glucans (mainly with β-1,3-linkages but also with α-1,4- and β-1,6-linkages) and that the mucilage is also composed of glucans. “Onozuka” R-10 cellulase is an impure enzyme mixture from the ascomycete *T. viride* that next to its main cellulolytic activities confers other enzymatic activities such as of hemicellulase, protease, amylase and pectinase (Serva Electrophoresis product information sheet; 43). *T. viride* enzyme preparations have further activities such as different glucanase activities that will assist in degradation of the chitin-glucan cell walls of the

C. cinerea spores (44,45). Sigma C6137 chitinase contains a chitodextrinase-chitinase, a poly(1,4-ß-[2-acetamido-2-deoxy-D-glucoside])-glycanohydrolase which at 37°C detaches chitobiose units from chitin and a *N*-acetylglucosaminidase-chitobiase which splits chitobiose into its monomers *N*-acetyl-D-glucosamine (Sigma product information sheet). We never experienced failure of protoplasting with newly bought batches of enzymes, but only with expired chitinase after storage for 5 years at -20°C. There is thus no urgent need for testing

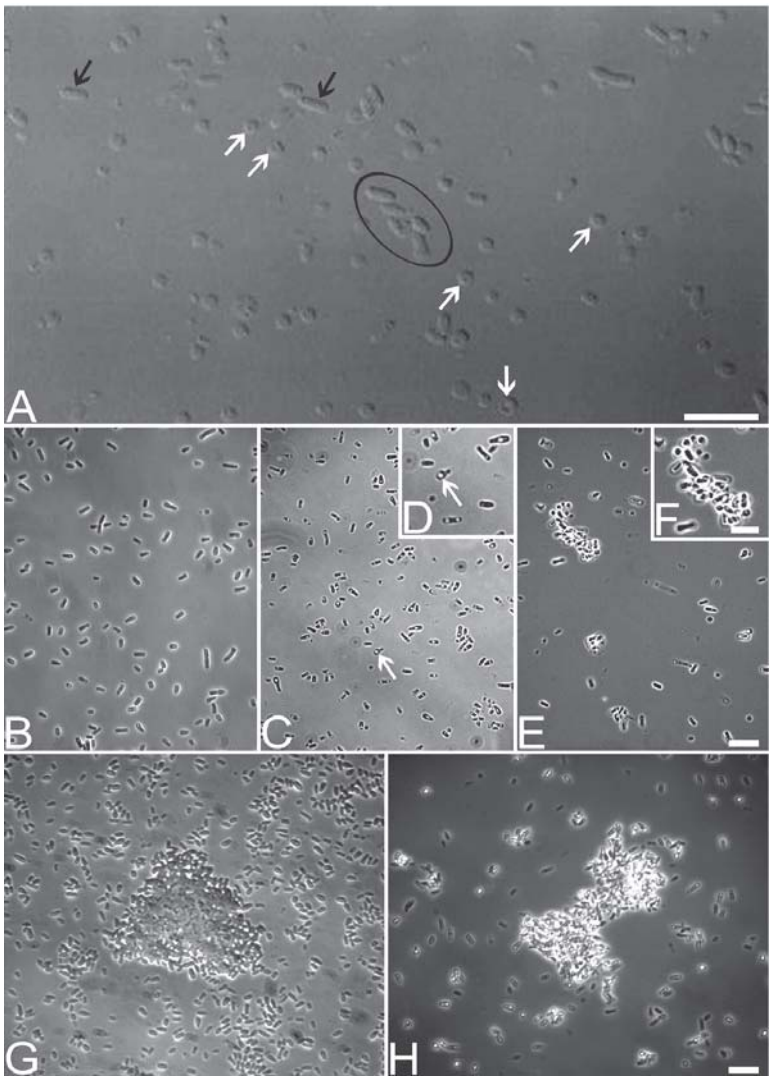

Fig. 4. Protoplasting of oidia. A. Protoplasts (with vacuoles; white arrows) and oidia (black arrows) of strain 7K17 (60% protoplasts; photographed by J.D. Granado) obtained with 2 mg/ml chitinase (encircled: a group of cells that start to aggregate with each other; size bar = 10 µm). B. Oidia suspension of strain FA2222 prior to addition of enzymes, C. and D. after 3 h incubation with 0.2 mg/ml chitinase (white arrows: cell releasing its protoplast), E. and F. respectively 6 h incubation with clumps of sphero- and protoplasts, G. after enzyme incubation and washing with MMC buffer, and H. after PEG addition (size bar for D and F = 10 µm; all others = 20 µm).

or applying other enzymes. However, Binninger *et al.* (4) replaced "Onozuka" R-10 cellulase with Novozyme 234 and succeeded in *C. cinerea* transformation with rates of 30 clones/µg pCc1001. In spite of this, Novozyme 234 was known to be very variable from batch to batch in quality of fungal protoplasting and in toxicity and it is now not anymore on the market (46).

The standard enzyme cocktail with 40 mg/ml cellulase and 1 mg/ml chitinase works very well in protoplast formation of up to 1-5 x 10^9 *C. cinerea* spores in 2 ml enzyme mix, with protoplasting rates of 50-70% in about 4 h incubation [(10); Fig. 4A]. However, each batch of spores is new and thus individual. Two transformation experiments performed with strain PG78 at two consecutive days gave so different results. In the first experiment after 4 h of incubation, a protoplasting rate of 75% was achieved and subsequent transformation with 1 µg pST17 mini-prep DNA resulted in total in 653 transformants. At the next day, protoplasting after 4 h was not satisfying and another 1 ml of enzyme of standard concentration was added for further 2 h of incubation when 50% of spores were protoplasted. Transformation of this second lot of protoplasts with 1 µg pST17 mini-prep DNA gave in total 382 transformants. An important question is when to best stop the digest. To decide this with confidence requires good experience of the researcher. Healthy protoplasts will look under the light microscope round and slightly more greenish than the spores, due to an altered light reflection at the protoplast membrane. First, protoplasts tend to be filled relatively equal with granular cytoplasma. Over the time, more protoplasts are generated and protoplasts might increase in size under development of large vacuoles (Fig. 4A). This stage likely reflects protoplasts where all cell wall has fully been degenerated. In *S. commune*, such type of naked protoplasts regenerates well (47). When *C. cinerea* protoplasts are harvested when this type appears, excellent transformation rates are usually observed. When further incubating with enzymes, this might not be anymore the case.

Cells in absence of enzyme are well suspended (Fig. 4B). When protoplasting spores of *C. cinerea*, after some time of enzyme incubation, cells still covered by cell walls start to clump together and with available protoplasts (Fig. 4A,E,F). We believe that this behaviour is induced by enzymatic alterations of the cell walls, marking partial cell wall digestion. The appearance of these clumps can also be used as a good indication that the cells are ready for transformation. In other fungi, protoplast regeneration might be accelerated when the cell wall is not completely degraded and transformation rates can be increased when using spheroplasts with cell wall remnants instead of naked protoplasts (48-50). Our more recent experiments show that chitinase concentrations might be reduced to 0.1 mg/ml for digestion of about 10^8 cells in 2 ml (in total 0.1 to 0.16 U) with the consequence, that protoplasting slows down (Fig. 4B-F). We observed in different experiments with strain FA2222 after 3 h of incubation regularly about 5% of protoplasted spores. Many other cells are then at a stage close to release the protoplast from the cell wall. Cells often swell at one end at which the protoplast finally escapes (Fig. 4C,D). With further incubation, 30-35% protoplasting can be observed after 6-7 h. At this stage, protoplasts and partially digested spores (spheroplasts) clump in groups together (Fig. 4E,F). Nevertheless of the lower rate of protoplasting, such sphero-/protoplast mixtures might be harvested for transformation. In 10 independent experiments with strain FA2222, 34 to 208 [in average 96 ± 69] *trp1*$^+$ transformants per 1-2 x 10^7 cells and 1 µg pCc1001 were so obtained.

Not unexpected, the time required for protoplasting is much influenced by the amount of chitinase added to the spores. Binninger *et al.* (1) digested spores in 2.5 h in 1 ml enzyme mix with 20 mg cellulase and 1 mg chitinase, stated by the authors to represent 4 U. Different batches of Sigma C6137 chitinase differ in total U/mg

which, in our hands, ranged between 0.5-0.8 U/mg enzyme powder. The standard procedure (10,23) with in total 80 mg cellulase and 2 mg (= 1-1.6 U) chitinase takes about 3 to 4 h (occasionally up to 5 h) for adaequate protoplasting. Overdigestion of the protoplasts might easier be controlled in longer incubation times. Since the times of incubation might well be used such as to prepare regeneration agar or, if required, also DNA, a longer incubation time might be of comfort for the experimenter. The steps following upon spore digestion are not as time consuming and the whole procedure can still well be performed within a working day when only 0.2 mg chitinase is applied.

Protoplasting is stopped by adding MMC buffer and subsequent centrifugation. The pellets of protoplast-spore mixtures are pearly with a slimy appearance. The supernatant is carefully poured off, thereby trying not to loosen the pellet at the bottom of the tube. If this nevertheless happens, it is advisable not to try to pipet the remaining liquid away but to add further MMC for another round of centrifugation to bring the Ca^{2+} concentration to 25 mM. The Ca^{2+} in the buffer causes the protoplasts to better stick to each other (Fig. 4G) and in consequence to better pellet. Also after the last centrifugation (after a second wash) it is better to carefully pour off the buffer with some MMC liquid remaining with the protoplasts in the tube. Trying to take off the rest with a sterile pipette tip will only cause dispersal of protoplasts when sucking the liquid and with it loss of protoplasts. The final pellet size and the left MMC volume will determine how much extra MMC might be added to the protoplasts. As a rule of thumb, about 10^6-10^7 cells should be present per 50 µl protoplast suspension (1,10,23) used in the next step for transformation. Note that it is not the exact number of cells that will guarantee the success in transformation but the individual valuation of the quality of protoplasts by the researcher. An additional dilution of the suspension, below 10^6 cells per 50 µl, should better be avoided. If more protoplasts are required for more transformation samples, more than one plate should be harvested. In the case that an excess of protoplast suspension exists, it is possible for later use to store the protoplasts overnight at 4°C or for a longer time at -80°C by addition of 50 µl sterile PEG/Ca^{2+} solution per 50 µl cell suspension.

Transformation: Generally, 1 µg mini-prep DNA per plasmid can directly be used in transformation. Application of larger DNA quantities (up to 50 µg plasmid DNA) does not necessarily lead to higher transformation efficiencies (1,7,15). The protoplasts are transformed by incubation with Ca^{2+} and PEG in ice at cold temperature and a following heat shock. Addition of Ca^{2+} and of PEG promotes cell agglomeration (Fig. 4G,H). Strengthened by the sudden temperature shift, Ca^{2+} and PEG are believed to take influence on the physiological properties of cell membranes in sphero- and protoplast transformation. Ca^{2+} and PEG may help to attach DNA to the cell surface and may enhance the permeability of the membrane and promote DNA internalization. However, in no fungus their exact function is so far understood (51,52).

Upon transformation and addition of 500 µl PEG/Ca^{2+} for further membrane interactions, addition of 1 ml sterile STC buffer (stabilized with 1 M sorbitol) dilutes the viscous PEG-protoplast mixture which eases the following plating on sucrose-stabilized regeneration medium. Cell wall regeneration needs time in an osmotically stabilized environment. It is therefore important that the protoplasts are brought onto plates in sufficient amount of stabilized liquid and that the liquid last a few days on the plates. Therefore at least 300 µl, better up to 400 µl of STC buffer-diluted protoplast solutions should be plated per Petri dish. Usually, one transformation sample results in four plates for regeneration. During plating, care should be taken for not diluting the sugar solution, i.e. that mixing is avoided with any condensate water that possibly accumulated on the plastic edges of the freshly poured Petri

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dishes. Plates must not be dried if such condensation is present to avoid disturbing the osmotic balance and reducing the a_w value in the agar.

Regeneration: Regeneration of protoplasts is done by incubation at 37°C. Important here is a first phase of keeping the agar surface of the regeneration plates wet for 2 to 3 days with a liquid layer. Thereafter, the plates should be shortly dried to a level at which the liquid layer is just gone away. It is to note that too extensive drying can negatively influence the further growth of the clones. With dryer plates, few transformants tend to grow. In some of our transformation experiments of strain FA2222 and pCc1001, regeneration plates dried already in the box during the first 2 days of incubation. In two different examples, only 10, 12, 6, and 3 (in total 31) transformants, respectively 15, 8, 2, and 3 (in total 28) clones could be harvested at the days 4 to 7 of incubation. This was not much different in a parallel cotransformation with pCc1001 and pYSK7 where 26, 10, 11, and 1 (in total 48) clones grew on the plates. In another experiment with FA2222 and pCc1001 as a typical example where the liquid was kept until active drying of the plates´ surfaces at day 3 of cultivation, 49, 78, 51 and 18 (in total 196) transformants were harvested at days 4 to 7 of incubation. It remains thus important to keep the a_w value in the agar as high as possible. In the protocol of Granado *et al.* (10) an agar concentration of 1.2% is used. A reduction to 1.0% can assist to keep a high a_w value and will still give the agar a required strength for plating.

Removing the liquid from the surface of regeneration plates after the initial three days of incubation reduces the risk of spreading bacterial contaminations. Even a single bacterial cell on a regeneration plate can spoil the whole transformation by rapid cell divisions and by the easy dispersal of the increasing number of bacterial cells in the liquid over the whole plate (Fig. 5A). As a further advantage, drying of the plates enhances the oxygen supply for the fungus to improve growth. Also, the contours of the small light-coloured fungal colonies become more clearly visible within the agar (Fig. 5B).

Picking emerging clones should be done as fast as possible upon appearance of transformants in order to avoid them to grow into each other. Small colonies and colony edges can best be recognized by holding the plates upright towards light (evenly distributed and not too strong light, such as natural day light from a window) whilst cutting out agar pieces with mycelium with a sharp tungsten needle. Alternatively, very small colonies might be

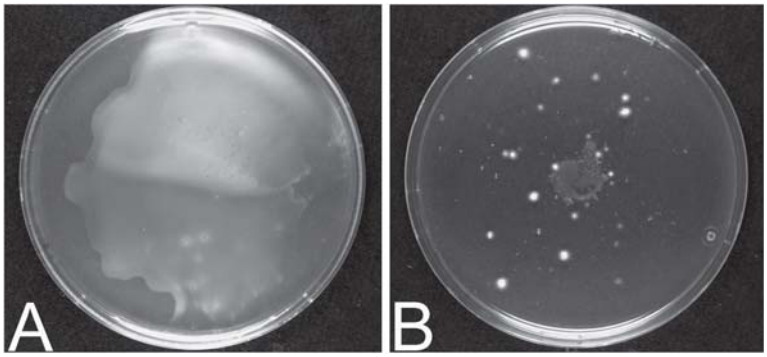

Fig. 5. Plates with regeneration agar after plating of transformation mixtures of strain FA2222 with pCc1001 and pYPH3 and 4 days incubation at 37°C with A. bacterial contamination and B. young *trp1*$^+$ transformants on regeneration medium made up with 1% agar.

harvested with the needle while observing the colonies under a binocular with the plate illuminated from below from an indirect light source. For further cultivation and appearance of more transformants, care has to be taken that from all colonies all material is removed since any remaining hyphal debris will further grow into a new colony. All picked clones should be transferred to suitable selection medium, usually minimal medium (with specific supplements added if required). Per plate of fresh medium, about 15 transformants might be inoculated (using a grid for equal distribution) to grow them for up to 2 days at 37°C without a danger that colonies grow into each other.

Conclusions

Transformation of *C. cinerea*, first presented by Binninger *et al.* in 1987 (1), is an essential tool for molecular analysis of this fungus. In this paper, we discuss important aspects for success in the transformation – DNA sources and quality, fungal cultivation and age of cultures, the procedure of enzymatic protoplasting and quality criteria of protoplasts, and best handling in regeneration. Well understanding details in the protocol can help to avoid failure in handling. Transformation of *C. cinerea* needs some practice. In particular, a good sense needs to be developed for recognition of high quality protoplasts.

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Anti-inflammatory and Antioxidant Potential of α -Mangostin

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Abstract

The anti-inflammatory activity was evaluated using acute and chronic inflammatory models like carrageenan induced paw oedema and cotton pellet induced granuloma models respectively. Oral administration of α -Mangostin showed dose-dependent and significant anti-inflammatory activity both in acute and chronic phases of inflammation. Antioxidant properties of α-Mangostin analyzed by DPPH, nitric oxide, superoxide radical scavenging assays showed enmarked free radical scavenging activity of α-Mangostin. Lipid peroxidation was also drastically inhibited by α-Mangostin in concentration dependent manner and showed an IC_{50} value of 900μg/ml. The present findings clearly validates the traditional use of α -Mangostin by confirming the anti-inflammatory and antioxidant potential of α-Mangostin.

Keywords: α-Mangostin, Xanthone, Anti-inflammation, Lipid peroxidation and Mangosteen.

Introduction

α-Mangostin, a xanthone derivative, is a major bioactive compound found in the fruit hull of Mangosteen and belongs to *Garcinia mangostana* tree of South East Asia. The whole Mangosteen fruit especially the xanthone packed pericarp has been used traditionally to treat a variety of health disorders. Medicinal properties of α-Mangostin include usage against trauma, diarrhea, gonorrhea, bladder infections and skin infections (1).

Inflammation is considered as a primary physiological defense mechanism that helps the body to protect against infection. The mechanism of inflammation is linked to release of reactive oxygen species (ROS) such as superoxide (O_2), hydroxyl (OH^-) and peroxy radicals (ROO^-) from activated neutrophils and macrophages. The ROS play an important role in the pathogenesis of various diseases and in the propagation of inflammation by stimulating release of cytokines and interferon-γ. Thus free radicals are important mediators that provoke or sustain inflammatory processes and conse-quently their neutralization by antioxidants and radical scavengers can attenuate inflammation (2).

The inflammatory response occurs in three distinct temporal phases, each apparently mediated by different mechanisms: i) an acute phase characterized by transient local vasodilation and increased capillary permeability, ii) a delayed sub-acute phase characterized by infiltration of leucocytes and phagocytic cells and iii) a chronic proliferative phase, in which tissue degeneration and fibrosis occur. However, chronic inflammation can also lead to a number of diseases, such as hay fever, rheumatoid arthritis, arteriosclerosis, myocarditis and cancer (3). The goal of present study is the validation of the traditional use of α-Mangostin and to determine the *in vivo* anti-inflammatory and *in vitro* antioxidant potential of 40% HPLC purified α-Mangostin.

Materials and Methods

Plant material: The 40% HPLC purified α-Mangostin was procured from INDFRAG Company, Bangalore, India; for analyzing anti-inflammatory and antioxidant properties.

Animals: Adult male albino rats (Wistar Strain) weighing between 150-200g were obtained from Bros Scientifics, Tirupati, India, and used for anti-inflammatory studies. All the animals were acclimatized for a week before use and were grouped in polyacrylic cages and maintained under standard laboratory conditions. The room temperature was maintained at 25 ± 2ºC with dark and light cycle of 14/10h. They were fed on commercial diet and water *ad libitum*. The Institutional Animal Ethical Committee approved protocols were followed for experimental analysis.

Acute toxicity test: Healthy rats, starved overnight, were divided into six groups of six animals in each group and fed with increasing doses (10,100, 250, 500, 1000, and 1500 mg/kg, b.w, p.o) of 40% HPLC purified α-Mangostin up to 14 days.

Anti-inflammatory activity of α-Mangostin

Carrageenan induced paw oedema in rats: The anti-inflammatory activity of α-Mangostin was determined by inducing acute inflammation by carrageenan in rats (4). For the experimental analysis, the rats were divided into five groups of six animals each. The first group (control) received normal saline (0.9% w/v, 3ml/kg, b.w, p.o). Second group with Indomethacin (10mg/kg) served as standard where as third, fourth and fifth groups were orally administered with low dose, mild dose and high doses (0.5, 5 and 10mg/kg) of α-Mangostin respectively with the help of an oral catheter. After 1h of drug treatment, a subplantar injection of 1% Carrageenan solution was administered in the left hind paw of rats. The volume of paw oedema was measured with Plethysmometer (UGO Basile, USA) after 3h of injections. The average paw volume was measured and compared with control and standard groups. The percentage of paw oedema inhibition was calculated using the formula;

Inhibition of oedema (%) = Oc-Ot / Oc x 100.

Where, 'Oc' is oedema volume of control group and 'Ot' is oedema volume of treated groups.

Cotton pellet induced granuloma in rats: The cotton pellet method is frequently used to evaluate the chronic phase of inflammation. The rats were divided into five groups of six animals in each group. The rats were anaesthetized by ether and sterile cotton pellets weighing 10mg were implanted subcutaneously into the groin region of each rat. The first group referred as control received normal saline (0.9% w/v, 3ml/kg, b.w, p.o), Second group served as standard, received Indomethacin (10mg/kg), where as third, fourth and fifth groups received low dose (0.5mg/kg), mild dose (5mg/kg) and high dose (10mg/kg) of α-Mangostin respectively with an oral catheter. The α-Mangostin treatment was continued for seven consecutive days from the day of cotton pellet implantation (5). After the completion of 7 days, i.e., on the beginning of 8th day, the animals were anaesthetized with ether and the pellets along with the granuloma tissue formed around were removed carefully and freed from extraneous tissue. Then the wet pellets were dried in an oven at 60ºC for 24h to constant weight for measuring the granuloma formation. The percent inhibition of granuloma formation was calculated by using the formula;

Inhibition of granuloma (%) =Gc-Gt / Gc x 100.

Where, 'Gc' is granuloma tissue weight in control group and 'Gt' is granuloma tissue weight in treated groups.

In vitro antioxidant study of α-Mangostin

DPPH radical scavenging assay: The antioxidant activity of α-Mangostin was analyzed based on the scavenging activity of stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical (6). All experiments were repeated thrice. Different concentrations (62.5-1000μg/ml) of α-Mangostin

in ethanol (0.05ml) were added to methanolic solution of DPPH (200μM). The mixture was shaken and allowed to stand at room temperature for 30min and the absorbance was measured at 517nm using methanol as blank on UV-VIS spectrophotometer (Shimadzu, Germany). Ascorbic acid was used as the standard. The scavenging activity is expressed as the percentage of inhibition at different concentrations calculated by using the formula and IC_{50} was determined by linear regression analysis.

Inhibition (%) = Absorption of control-Absorption of test / Absorption of control × 100.

Nitric oxide radical scavenging assay: Sodium nitroprusside in an aqueous solution at physiological pH, spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that were measured at 546nm using Griess reagent (7). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. In order to determine the scavenging activity of α-Mangostin, sodium nitroprusside (5mM) in standard phosphate buffer solution was mixed with different concentrations of α-Mangostin (62.5-1000μg/ml) and the tubes were incubated at 25 ±2°C for 5h. Control was maintained by adding an equal amount of phosphate buffer. About 0.5ml of incubated solution was mixed with equal amount of Griess reagent and the color developed was measured at 546nm. The percentage inhibition was calculated by using the same formula as given above and IC_{50} was determined.

Scavenging of superoxide radical: The scavenging activity towards the superoxide radical (O_2^{--}) was measured in terms of inhibition of generation of O_2^- (8) using alkaline dimethyl sulphoxide (DMSO) method (9). Potassium superoxide and dry DMSO were allowed to stand in contact for 24h and the solution was filtered just before use. The filtrate (200μl) was added to 2.8ml of an aqueous solution containing nitroblue tetrazolium (56μl), ethylene diamine tetra acetic acid (10μl) and potassium phosphate buffer (10mM). Then various concentrations (62.5-1000μg/ml) of α-Mangostin in ethanol (1ml) were added and absorbance was recorded at 560nm against a blank. The percentage inhibition was calculated by using the same formula as given above and then IC_{50} was determined.

Lipid peroxidation assay: The extent of Lipid peroxidation in rat brain homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) (10). Different concentrations of α-Mangostin (62.5-1000μg/ml) in ethanol were individually added to the brain homogenate (0.5ml). This mixture was incubated with 0.15M KCl (100μl). Lipid peroxidation was initiated by adding 100μl of 15mM $FeSO_4$ solution and the reaction mixture was incubated at 37ºC for 30 min. After incubation, the mixture was added to 1ml of solution containing equal volume of TBA (thiobarbituric acid): TCA (trichloroacetic acid). Then the mixture was heated at 80ºC for 20 min after the addition of 1ml of butyrated hydroxyl toluene and was centrifuged after cooling to room temperature. The absorbance of supernatant was read at 532nm against blank. The percentage of Lipid peroxidation inhibition was measured by using the same formula as given above and then IC_{50} was calculated.

Statistical analysis: All the data are triplicates of independent experiments reported as Mean ± SEM (Standard Error of Mean). Statistical significance was assessed by *t*-test using InStat software.

Results

Acute toxicity test: The oral doses of α-Mangostin up to 1500mg/kg did not produce any evident sign of toxicity and mortality in rats when observed up to 14 days since administration. Thus, the median lethal dose (LD_{50}) was determined to be higher than the maximum (1500mg/kg) dose tested.

Anti-inflammatory activity of α-Mangostin: In experimentally induced paw oedema by carrageenan, α-Mangostin showed significant

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anti-inflammatory activity in dose dependant manner by restricting the paw oedema volume to 0.883±0.012 at 10mg/kg b.w, p.o after 3h of treatment compared to control group (Table 1). The inhibition of paw oedema was clearly observed in α-Mangostin (10mg/kg) treated group compared to control group (Fig.1). The maximum percentage of paw oedema inhibition of 40.21% was observed at 10mg/kg of α-Mangostin (Fig. 2) in comparison with standard Indomethacin (10mg/kg).

In cotton pellet induced granuloma method dry weight of the cotton pellets were taken as measure of granuloma formation, α-Mangostin significantly reduced the granuloma formation at 10mg/kg b.w, p.o (Table 2). The formation of granuloma around subcutaneously implanted cotton pellets in the groin region of rats was observed in control group whereas it was not observed in 10mg/kg of α-Mangostin treated group (Fig. 3). The maximum inhibition of 66.71% at 10mg/kg of α-Mangostin was observed where as Indomethacin showed a maximum of 50.57% inhibition of granuloma at 10mg/kg (Fig. 4).

Antioxidant potential of α-Mangostin: The DPPH was widely used as a model system to investigate the scavenging activities of natural compounds. As shown in Table 3 the percentage of inhibition was found to be 95% at 1000μg/ml

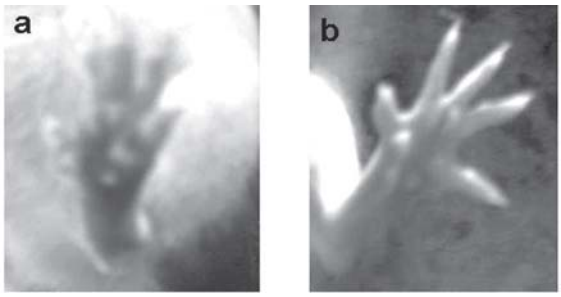

Fig. 1. The anti-inflammatory activity of α-Mangostin was evaluated in acute phase of inflammation in rats. The acute inflammation was experimentally induced by carrageenan showing paw oedema in control group (a) and inhibition of paw oedema in 10mg/kg b.w of α-Mangostin treated group (b) after 3h of induction.

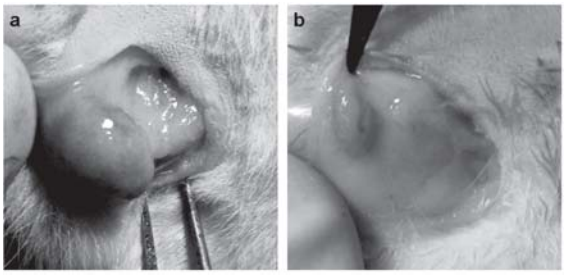

Fig. 3. The anti-inflammatory activity of α-Mangostin was evaluated in chronic phase of inflammation in rats. The chronic inflammation was induced by cotton pellet showing formation of granuloma around subcutaneously implanted cotton pellets in the groin region of rats in control group (a) but not in 10mg/kg b.w of α-Mangostin treated group (b) after 7 days of induction.

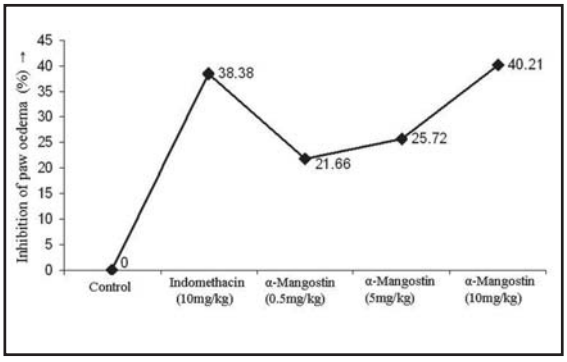

Fig. 2. Anti-inflammatory activity of α-Mangostin evaluated by carrageenan induced acute inflammation in rats.

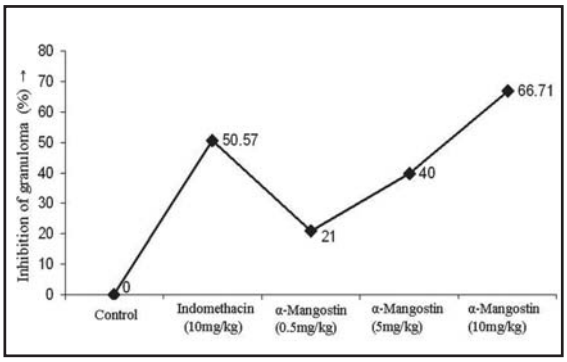

Fig. 4. Anti-inflammatory activity of α-Mangostin evaluated by cotton pellet induced chronic inflammation in rats.

Table 1. Effect of α-Mangostin on paw oedema induced by carrageenan in rats

Group / Treatment	Dose (mg/kg,p.o)	Mean paw oedema volume (ml) ± SEM
Group 1 / Control	—	1.477±0.117
Group 2 / Indomethacin	10	0.910±0.070*
Group 3 / α-Mangostin	0.5	1.157±0.042*
Group 4 / α-Mangostin	5	1.097±0.038*
Group 5 / α-Mangostin	10	0.883±0.012*

Data given are mean of three replicates ± SEM
*p<0.001 when compared to control based on a Student's't' test.

Table 2. Effect of α-Mangostin on cotton pellet induced granuloma in rats

Group / Treatment	Dose (mg/kg,p.o)	Dry weight of cotton pellet (mg) ± SEM
Group 1 / Control	—	70.0±0.13
Group 2 / Indomethacin	10	34.6±0.03*
Group 3 / α-Mangostin	0.5	55.3±0.09*
Group 4 / α-Mangostin	5	42.0±0.05*
Group 5 / α-Mangostin	10	23.3±0.15*

Data given are mean of three replicates ± SEM
*p<0.01 when compared to control based on a Student's't' test.

and 40% at a concentration of 62.5µg/ml. The IC_{50} value of α-Mangostin was found to be at 100µg/ml. This study showed that the α-Mangostin has the proton-donating ability and could serve as free radical scavenger.

Nitric oxide scavenging effect of α-Mangostin was found to be 12% and 49% at 62.5µg/ml and 1000ìg/ml respectively. α-Mangostin showed an IC_{50} value at 1000µg/ml (Table 4). Superoxide scavenging effect of α-Mangostin was found to be 27% and 89% at 62.5µg/ml and 1000µg/ml respectively with an IC_{50} of 250µg/ml (Table 5).

In vitro induction of lipid peroxidation is a tool for measuring antioxidant potential of á-Mangostin. Dose-dependent protection against lipid peroxidation (Table 6) exhibiting the IC_{50} value at 900µg/ml was observed with α-Mangostin. This activity may be related to the H^+ ion donating capability of the α-Mangostin which can scavenge the peroxyl radical.

Discussion

In the present study, systematic approach was made to find out the efficacy of α-Mangostin against inflammation so as to exploit it as herbal anti-inflammatory agent. Carrageenan induced rat paw oedema is commonly employed experimental animal model for evaluating the anti-inflammatory activity of natural compounds (11). It is well known that carrageenan induced oedema is multimediated phenomenon characterized by biphasic response with the involvement of different inflammatory mediators. The early phase (1-2h) is mediated by histamine and serotonin and late phase (3-4h) is mediated with the release of prostaglandins (12). Our results indicate that the administration of α-Mangostin inhibited the oedema during acute

Table 3. DPPH radical scavenging activity of α-Mangostin

α-Mangostin (μg/ml)	Percentage of inhibition*	IC_{50} (μg/ml)
62.5	40	
125	64	
250	68	100
500	70	
1000	95	

*Values are means (n=3)

Table 4. Scavenging effect of α-Mangostin on Nitric oxide radical

α-Mangostin (μg/ml)	Percentage of inhibition*	IC_{50} (μg/ml)
62.5	12	
125	15	
250	20	1000
500	30	
1000	49	

*Values are means (n=3)

Table 5. Scavenging activity α-Mangostin on Superoxide radical

α-Mangostin (μg/ml)	Percentage of inhibition*	IC_{50} (μg/ml)
62.5	27	
125	39	
250	54	250
500	59	
1000	89	

*Values are means (n=3)

Table 6. Inhibition of Lipid peroxidation by α-Mangostin

α-Mangostin (μg/ml)	Percentage of inhibition*	IC_{50} (μg/ml)
62.5	12	
125	19	
250	26	900
500	32	
1000	54	

*Values are means (n=3)

phase of inflammation probably by inhibiting the chemical mediators of inflammation.

The cotton pellet method is widely used to evaluate the proliferative components of chronic inflammation (13). Cytokines, such as IL-1 and TNFα, as well as growth factors influence proliferation of smooth muscle cells and fibroblasts and production of granuloma (14, 15). The weight of the wet cotton pellets correlates with transude material and the weight of dry pellet correlates with the amount of granuloma tissue formation. The Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the size of granuloma which results from cellular reaction by inhibiting granulocyte infiltration, preventing generation of collagen fibers and suppressing mucopolyssaccharides (16). In the present study, oral administration of α-Mangostin has been observed to inhibit the wet weight of cotton pellet in a dose dependent manner and the higher dose of α-Mangostin exhibited more inhibition of inflammation compared with the standard NSAID Indomethacin, which indicates that the proliferative phase was effectively suppressed by α-Mangostin. Several chronic human diseases associated with inflammation are characterized by over production of ROS (17). DPPH radical is scavenged by antioxidants through the donation of proton by forming the reduced DPPH. The color changes from purple to yellow after reduction can be quantified at 517nm (6). DPPH was used to determine the proton radical scavenging action of α-Mangostin, because it possess a proton free radical and showed a characteristic absorbance at 517nm. From the present study, it was found that α-Mangostin reduces the radical to corresponding hydrazine when it reacts with hydrogen donors.

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc., and is involved in the regulation of various physiological processes (18). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The α-Mangostin inhibited the nitrite formation either by competing with oxygen or with its

synthesis. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (19). Nitric oxide radical inhibition assay proved that α-Mangostin is a potent scavenger of nitric oxide.

Superoxide dismutase (SOD) continues to be an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 (20). The findings proved the strongest superoxide scavenging activity of α-Mangostin.

Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids and involves in the formation of lipid radicals leading to membrane damage (21). Ferrous ions can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA). The inhibition could be caused by the absence of ferrule-perferryl complex or by scavenging the hydroxyl radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating iron itself. The antioxidant activity of α-Mangostin was further confirmed by decreased production of MDA in the biomembrane of rat brain homogenate.

Conclusion

The administration of α-mangostin inhibited the oedema and granuloma formation during acute and chronic inflammatory conditions. The present study reveals that α-mangostin has significant anti-inflammatory and free radical scavenging activity. Further studies are needed to analyze the therapeutic potential of α-mangostin against chronic inflammatory diseases.

Acknowledgement

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Exposure to Metal Mixture of Lead and Arsenic Impacts Superoxide Dismutase Activity and Expression in Rat Brain

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Abstract

Neurotoxicity of individual metals is well investigated but that of metal mixture, an environmental reality, in the brain is relatively obscure. We investigated the combinatorial effect of metals (As and Pb) in brain regions such as cortex, cerebellum and hippocampus and compared the toxicity levels with individual metal exposed group. The studies were conducted to examine the alterations in SOD isoforms (SOD-total, Mn - SOD, Cu/Zn - SOD) because it is the first line of antioxidant defense and is highly efficient in protecting cells against oxidative stress by catalyzing the dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen. The results showed that metal mixture produced decrease in SOD isoforms and gene expression level in brain regions than individual metal exposure. Among the brain regions, the hippocampus showed greater decreases in Mn - SOD and Cu/Zn - SOD enzyme activities and gene expression levels than cerebellum and cortex. These effects were greater following metal mixture exposure as compared to individual metal exposure.

Keywords: Arsenic, Lead, SOD isoforms, Rat brain regions.

Introduction

Heavy metals including arsenic (As), cadmium (Cd), and lead (Pb) have received attention as both environmental contaminants and potential neurotoxicological hazards (2, 7, 15). Neurotoxicity of individual metals is well investigated but that of metal mixture (MM), an environmental reality, in the developing brain is relatively obscure. Studies with single metal exposure have demonstrated that heavy metals infiltrate the immature blood-brain barrier and accumulate in developing brain (22, 35, 37). Chronic exposure to As, even at a submicromolar concentration, promotes oxidative stress (11) and induces neuroglial damage in human brain (16). Chronic low-level exposure to inorganic Pb constitutes the more serious occupational hazards and health risk (36) Pb and As each have been shown to induce oxidative stress (23, 29, 33). Oxidative stress can trigger undesirable biological reactions leading to a wide variety of pathophysiological processes, including neuronal cell death and tissue injuries within the nervous system (25). While the exact mechanisms by which mixtures of Pb and As produce toxicity are still being elucidated (34), oxidative stress appears to play a central role. Oxidative damage induced by either Pb or As causes brain damage because the brain is believed to be particularly vulnerable due to its high oxygen consumption rate and high level of polyunsaturated fatty acids (PUFA) (4). Among the antioxidant enzymes, superoxide dismutase (SOD) is the potential target for various environmental toxicants and a

sensitive marker to assess the extent of oxidative damage. SOD is thought to be one of the first lines of antioxidant defense and is highly efficient in protecting cells against oxidative stress by catalyzing the dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen. This enzyme presents in three isoforms, i.e. extracellular superoxide dismutase (EC SOD), manganese containing superoxide dismutase (Mn - SOD) and copper-zinc contains superoxide dismutase (Cu/Zn - SOD) (38). Several studies reported the effect of As and Pb individually on the brain antioxidant enzymes but, none of the studies have reported the combined effect of As and Pb on antioxidant enzyme, SOD in various brain regions. This is critical because free radical generation and the capacity of detoxification mechanisms in specific regions of the brain will probably fluctuate during metal mixture exposure. Therefore, the Present study was aimed to examine the changes in the specific activity and expression of antioxidant enzyme SOD (SOD isoenzyme, Mn - SOD and Cu/Zn - SOD) in discrete brain regions of rats following exposure to combination of As and Pb.

Materials and Methods

Chemicals **:** Lead acetate and sodium arsenite used in this study were purchased from Sigma Chemical Company, St Louis, MO, U.S.A and all other chemicals were purchased from Merck, India.

Animals Exposure: One month old rats were housed in polypropylene cages (47 cm x 34 cm x 20 cm) containing sterile paddy husk (procured locally) as bedding material and maintained in the animal facility at 28 ± 2ºC and relative humidity 60 ± 10% with a 12 h light/day cycle. The animals were fed in the laboratory with standard pellet diet supplied by Sri Venkateswara Traders, Bangalore and water *ad libitum.* . The protocol and animal use were approved by Animal ethical clearance committee, S.V. University. Animals were randomly divided into four groups of six animals and treated for three weeks as follows.

Group I: Control (received normal water)

Group II: Arsenic (20 mg/kg, orally)

Group III: Lead (20 mg/kg, orally)

Group IV: Arsenic + Lead (10 mg/kg + 10 mg/kg, orally)

Preparation of mitochondrial fraction: Mitochondrial fractions of cerebral cortex, cerebellum and hippocampus were prepared following the method of Lai and Clark (20). Briefly, the tissue was homogenized in 5 volumes (w/v) of SET buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4). The homogenate was first centrifuge at 800 *g* for 10 min at 4°C, and then the supernatant was centrifuged at 10,000 *g* for 20 min at 4°C. Then the pellet of mitochondrial fraction was suspended in SET buffer.

Sample preparation for nondenaturing PAGE: The brain regions were homogenized using a Potter homogenizer with a glass pestle, in 9 volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. Homogenate was centrifuged at 30,000×*g* for 30 min at 4 °C and the resultant supernatants were aliquoted and stored at “80 °C for iso enzyme assay.

Analysis of SOD isoenzymes on non-denaturing PAGE: For the separation of SOD isoenzymes, nondenaturing PAGE was performed on 10% acrylamide slab minigels (MiniProtean II, Bio-Rad). SOD isoenzymes were detected in gels by the photochemical NBT (nitroblue tetrazolium) staining method (1). The different types of SOD were differentiated by performing the activity stains in gels previously incubated for 20 min at 25 °C in 50 mM potassium phosphate buffer, pH 7.8, containing either 50 mM KCN or 5 mM H_2O_2. Cu/Zn - SODs are inhibited by KCN and H_2O_2, Fe-SODs are resistant to CN^- but inactivated by H_2O_2, and Mn - SODs are resistant to both inhibitors (8).

Determination of activity: SOD activity was determined by using the epinephrine assay of Misra and Fridovich (27). At alkaline pH,

superoxide anion O^-_2 causes the autooxidation of epinephrine to adenochrome; while completing this reaction, SOD decrease the adenochrome formation. One unit of SOD is defined as the amount of extract that inhibits the rate of adenochrome formation by 50%. The reaction mixture in a final volume of 2.0 ml contained 1.76 ml of 0.05 M carbonate buffer (pH 10.2), 0.04 ml of 30 mM epinephrine (freshly prepared) and 0.2 ml of enzyme source. 1 mM potassium cyanide inhibited both Cu/Zn - SOD and extracellular SOD resulting only Mn - SOD activity only. Changes in absorbance were recorded at 480 nm, measured at 10 sec intervals for 1 min in a spectrophotometer. The enzyme activity was expressed as Units/mg protein.

RT-PCR (Reverse transcriptase PCR) Analysis: The expression of Mn - SOD and Cu/Zn - SOD was evaluated by RT-PCR. Total RNA was isolated from cerebral cortex, cerebellum and hippocampus using RNA-X press Reagent (HIMEDIA, India). The purity and concentrations of the RNA samples were assessed by OD 260/OD 280 spectrophotometric measurements and by agarose gel electrophoresis. The ratio of OD 260/OD 280 of all extracted RNA samples was between 1.8 and 2.0. RNA was transcribed into first-strand cDNA using revertAid first strand cDNA synthesis kit (Fermentas, India). The synthesized cDNAs were amplified by one cycle of 95^0 for 5 min and amplified by 30 cycles of PCR (denaturation at 94^0C for 1min, annealing at 56^0C for 1 min, extension at 72^0C for 2 min for Mn - SOD and denaturation at 94^0C for 1 min, annealing at 57^0C for 1 min, extension at 72^0C for 2 min for Cu/Zn - SOD).Final extension was at 72^0C for 5 min (Mn - SOD, Cu/Zn - SOD). The mRNA levels of the Mn - SOD and Cu/Zn - SOD were normalized against β-actin. Specific β-actin primers were used for the internal control to normalize the sample amounts. The sequences of oligonucleotide primers used for PCR amplification of Mn-SOD, Cu/Zn - SOD and positive control β-actin were as follows: Mn-SOD: Forward primer 5'-ACG CGA CCT ACG TGA ACAATCT -3' and Reverse primer 5'-CAG TGC AGG CTG AAG AGC AA -3'; Cu/Zn - SOD: Forward primer 5'-GAT TAA CTG AAG GCG AGC AT -3' and Reverse primer 5'-CCG CCA TGT TTC TTA GAG T -3'; β-actin: Forward primer 5' AGC AAG AGA GGCATC CTG AC-3' and Reverse primer 5'-GTG TACGA CCA GAG GCA TA-3'. The PCR products were separated by electrophoresis using 1% agarose gels stained with ethidium bromide to visualize cDNA products. Bands of each target transcript were visualized by ultraviolet transillumination and captured using a digital camera. ODs for each band were quantified by image J analysis software.

Data Analysis: The data obtained from six separate samples were expressed as mean ± SD. Data were analyzed by two-way Analysis of Variance (ANOVA) following Standard Statistical Software Package to compare the effects among various groups. The 0.05 level of probability was used as the criterion for significance.

Results

The individual and combined effect of As and Pb on total SOD activity in different brain regions was shown in Fig. 1. Total SOD activity in different brain regions decreased significantly following individual and combined exposure to As and Pb in different brain regions of rat. In control animals, highest SOD activity was observed in cortex (4.051) followed by hippocampus (3.119) and cerebellum (2.407). Both Pb as well as As exposure inhibited SOD activity but the inhibition was found to be greater following combined exposure. Among the individual metals, inhibition in SOD activity was higher with As compared to Pb. The inhibition of total SOD was greater and region specific in rats exposed to metal mixture of As and Pb. Maximum inhibition found in hippocampus (54.69 %), followed by cerebral cortex (49.49%), cerebellum (45.36%). The observed inhibition in SOD activity was significant at $P<0.01$.

Fig.2 and 3 depicts the individual and combined effect of As and Pb on Cu/Zn - SOD and Mn - SOD levels in different brain regions.

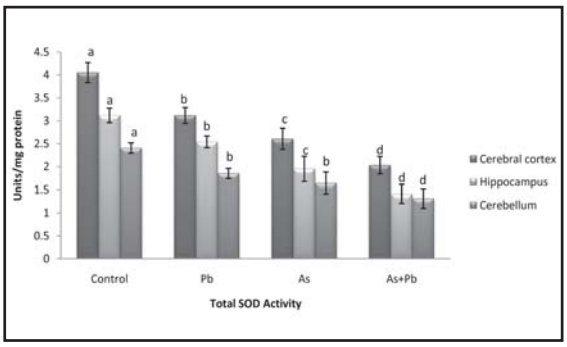

Fig.1. Total SOD activity was assayed in mitochondrial fraction of cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats. The enzyme activity was expressed as units/mg protein. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other (p<0.05).

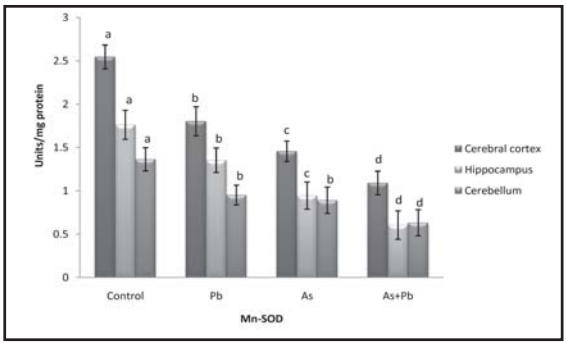

Fig.2. Mn – SOD activity was assayed in mitochondrial fraction of cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats. The enzyme activity was expressed as units/mg protein. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other (p<0.05).

In control animals, highest Cu/Zn SOD and Mn SOD activity was observed in cortex (1.504, 2.547) followed by hippocampus (1.356, 1.763) and cerebellum (1.042, 1.365). Pb and As exposure inhibited both the isoforms of SOD but the inhibition was found to be greater in combined metal exposure. The inhibition of Cu/Zn - SOD and Mn - SOD was found to be maximum in hippocampus (40.48%, 65.62%) than cortex (37.36%, 57.12%) and cerebellum (34.45%, 53.69%). The observed inhibition in SOD activity was significant at P<0.01.

Qualitative changes in SOD isoenzymes banding pattern were observed in three different brain regions. After incubation with specific inhibitor (KCN), it was found that the cathodic band corresponded to Mn SOD, whereas the anodic band corresponded to Cu/Zn - SOD according to their increasing electrophoretic mobility. Optical density of each band was quantified by image J 5.0 software and the data of Cu/Zn - SOD and Mn - SOD were shown in Figure 4 and 5. The optical densities were proportional to the concentration of enzyme present in the sample. The specific activity of Cu/Zn - SOD and Mn - SOD was decreased in all treated groups. Cu/Zn - SOD and Mn - SOD from combined metal exposure showed lesser activity in all three brain regions when compared to control and individual metal exposed groups. The decrease in activity of Cu/Zn - SOD and Mn - SOD was higher in hippocampus (46.44%/

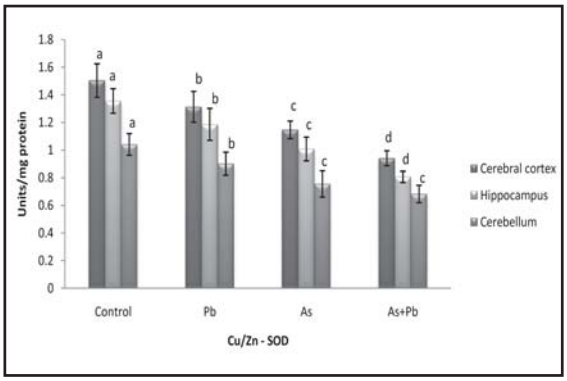

Fig.3. Cu/Zn–SOD activity was assayed in mitochondrial fraction of cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats. The enzyme activity was expressed as units/mg protein. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other (p<0.05).

49.67%) than cerebral cortex (40.1%/36.3%) and cerebellum (37.4%/34.03%). The inhibition observed in both Cu/Zn - SOD and Mn - SOD was significant at P<0.01(Figs. 4 and 5).

Cu/Zn - SOD and Mn – SOD gene expression levels were examined in animals subjected to both individual and combined exposure of As and Pb and the data are shown in Figs. 6 and 7. Significant changes were observed in all groups when compared to controls. The decrease observed in Cu/Zn - SOD and Mn – SOD gene expression levels following combined metal exposure was greater than individual metal exposures (As or Pb). Regional variations were observed in Cu/Zn - SOD and Mn – SOD gene expression in brain. Maximum decrease was observed in hippocampus (35.83% / 63.08) followed by cerebral cortex (32.62% / 52.6%) and cerebellum (27.97% / 42.56%). Decrease observed in gene expression levels were found to be significant at p<0.01. Among individual metal exposure (As and Pb), the expression levels of Cu/Zn - SOD and Mn –SOD were lower in As exposed brain (Figs. 6 and 7).

Discussion

Enhanced production of free radicals and inhibition of antioxidant enzymes have been suggested as possible mechanism to explain As and Pb induced oxidative stress (24, 28). SOD is the potential target for As and Pb toxicity because this antioxidant enzyme depends on essential trace elements for proper molecular structure and activity (12, 26). Our study demonstrated the activity of Cu/Zn - SOD and Mn – SOD is region specific in brain regions (cortex, hippocampus and cerebellum). Hippocampus is more vulnerable to combined exposure of As and Pb compared to cortex and cerebellum. The susceptibility of hippocampus indicates that it is an important target of neurotoxic agents and it accumulates metals to a greater degree than other parts of brain (Stoltenburg-Didinger 1994). The greater decrease in Mn - SOD activity than Cu/Zn - SOD in the mitochondrial fraction of brain regions is due to localization of Mn-SOD predominantly in mitochondria. It is a *Mr* 85,000 tetrameric enzyme located in the mitochondrial matrix, is the principal

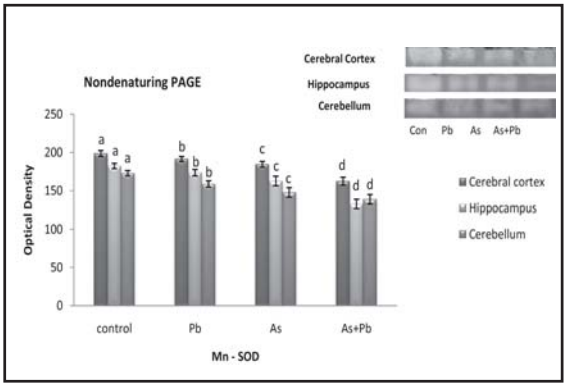

Fig. 4. Qualitative changes of Mn – SOD activity in crude extracts of cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats on non denaturing polyacrylamide gels. The optical density readings were obtained by processing the image with image J software. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other (p<0.05).

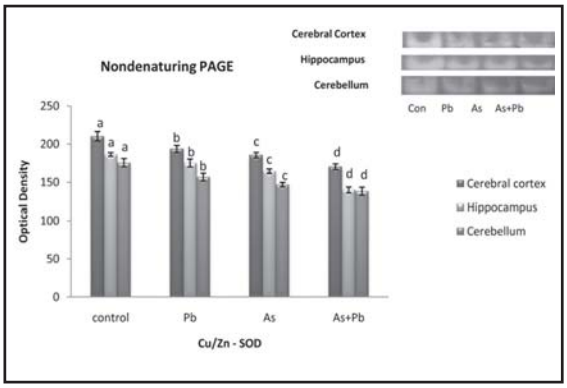

Fig. 5. Qualitative changes of Cu/Zn–SOD activity in crude extracts of cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats on non denaturing polyacrylamide gels. The optical density readings were obtained by processing the image with image J software. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other (p<0.05).

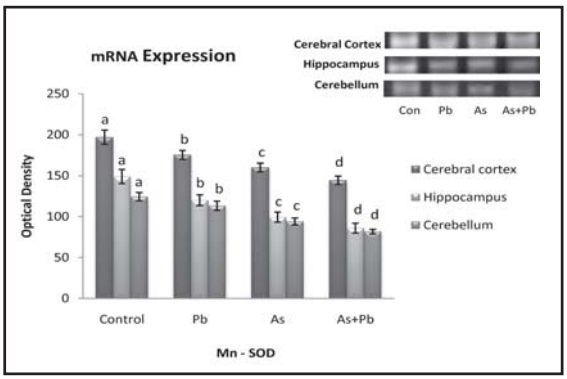

Fig. 6. Mn – SOD gene expression in cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats. The optical density readings were obtained by processing the image with image J software. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other ($p<0.05$).

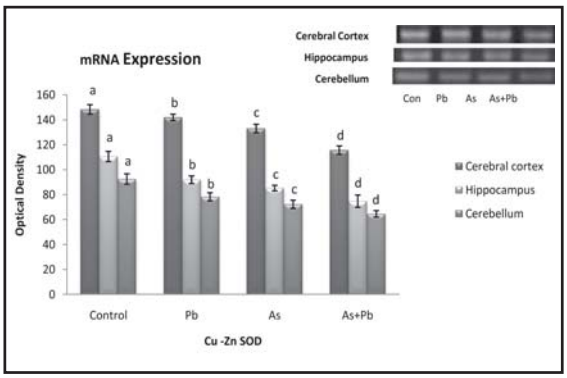

Fig. 7. Cu/Zn–SOD gene expression in cerebral cortex, cerebellum and hippocampus following individual and combined exposure of As and Pb to one month old rats. The optical density readings were obtained by processing the image with image J software. Values represent mean ± S.D. (n=6). Values that share same superscript do not differ significantly from each other ($p<0.05$).

scavenger for superoxide in mitochondria (9). Crude extract were used to know the activity of Mn - SOD and Cu/Zn - SOD on native polyaccylamide gels. Maximum decrease in the activity of Mn - SOD and Cu/Zn - SOD was observed in hippocampus than other regions following combined exposure. Higher levels of Cu/Zn - SOD activity protect neurons and other cell types against oxidative stress (13). Decrease in the activity of Cu/Zn - SOD has been shown to accelerate spontaneous cell death (32). A significant decrease in the activity of Cu/Zn - SOD and Mn – SOD following combined exposure might be due to greater affinity of As and Pb towards sulfhydryl groups and the changes in the confirmation of enzymes. The pathogenetic effects of As and Pb is multifactorial since it directly interrupts the activity of enzymes, competitively inhibits absorption of important trace minerals and deactivates antioxidant sulfhydryl pools (6). It is well known that over expression of Mn - SOD is neuroprotective and that changes in Mn - SOD expression can cause neuronal cell death in response to oxidative stress (10, 18). Following combined exposure to As and Pb, Mn - SOD and Cu/Zn - SOD gene expression was down regulated in hippocampus. As and Pb may interfere with signal transduction pathways and affects the transcriptional factors which are responsible for regulation of Mn - SOD and Cu/Zn - SOD but the exact mechanism for the down regulation of Mn - SOD and Cu/Zn - SOD gene expression are still not known. Decrease in Mn - SOD expression might be caused by STAT3 deactivation. Promoter analysis revealed the sites most abundant for the potential of signal transducer and activator of transcription 3 (STAT3) binding in the promoter region of mouse Mn - SOD genes. STAT3 is a transcription factor, as well as an intracellular signal transducer, activated by cytokines, growth factors, and receptor- or nonreceptor-tyrosine kinases (5, 21). Tyrosine phosphorylation of STAT3 at Y705 is necessary for STAT3 activation. Phosphorylated STAT3 forms dimers, translocates to the nucleus, binds to the specific promoters of target genes, and induces gene expression (3). Down regulation of Cu/Zn - SOD expression might be deactivation of PI3K/Akt. PI3K/Akt axis regulates the expression of Cu/Zn

- SOD via the activation of NF-*k*B (17). Shi *et al.* (30) provided evidence that As generates free radicals that leads to cellular damage and death through the activation of oxidative sensitive signaling pathways. ROS play a significant role in altering the signal transduction pathway and transcription factor regulation. Numerous reports have indicated that As affects transcriptional factors either by activation or inactivation of various signal transduction cascades (14, 19). Thus, the finding of our present study reports a definite synergistic trend of simultaneous exposure to As and Pb as compared to the effect of individual exposure. It can therefore be suggested that exposure to As and Pb not only inhibit normal functions of Mn - SOD and Cu/Zn - SOD sensitive markers of oxidative stress, but also that a simultaneous exposure has a synergistic effect. Further, these changes are brain region specific, with hippocampus exhibiting greater vulnerability.

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Construction of a Vector for the Constitutive Expression of Human Papilloma Virus type 16 Genes in *Salmonella Enterica* Serovar Typhi Strain Ty21a

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Abstract

Salmonella enterica Serovar Typhi strain Ty21a (*S*. Typhi Ty21a) is the only licensed live-attenuated oral vaccine against Typhoid fever in humans. It is also an oral delivery vehicle for heterologous antigen administration. This article describes the development of a constitutive expression vector, pSalEx, which is derived from pBR322 based vector, pProEx-HTb, for inducible expression of genes in *E. coli*. Repressor element, *lac* I, was removed from pProEx-HTb using PCR and restriction digestion. The pSalEx vector is smaller compared to the parent vector, pProEx-HTb, allowing larger inserts to be cloned and expressed. Expression of the major capsid protein (L1) of HPV16 under P_{trc} promoter of pSalEx was comparable to the constitutive expression of HPV16L1 from the pFS14nsd under the strong P_{tac} promoter. We demonstrate that pSalEx may be used for expression of heterologous genes in Ty21a and generally in other strains and species of bacteria belonging to the family *Enterobacteriaceae.*

Keywords : HPV 16 genes, pSalEx, *S*. Typhi Ty21a, Constitutive expression.

Introduction

Effective oral vaccines offer significant advantages over the conventional parenteral vaccines. The notable advantages of oral vaccine are the ease of administration with little or no intervention from medically skilled personnel and less stringent regulatory compliance compared to parenteral vaccines. Development of recombinant oral vaccines based on bacterial delivery vehicles that express either heterologous antigens or carry DNA for delivery into host cells are gaining prominence the world over. Bacterial vectored vaccine development has mostly been centered on *Listeria, Shigella, Lactobacilli* and *Salmonella* (1, 2, 3, 4). The live-attenuated Typhoid fever bacterial vaccine *Salmonella enterica* serovar Typhi strain Ty21a has been reasoned as a logical choice for heterologous antigen delivery (5, 6, 7). The attenuation of the bacteria generated by chemical mutagenesis has proven to be extremely safe while being fairly immunogenic. There has been no report of any major adverse affect from the millions of vaccinated populace in the nearly three-decade long use of the vaccine (8). Several target antigens of various pathogens have been expressed in the *S*. Typhi Ty21a. The recombinant *S*. Typhi has been proven efficacious in eliciting immunological responses specific to the expressed antigens (7, 9, 10, 11). Some of the promising candidate vaccines have progressed to clinical trials in humans (12, 13, 14).

Vectors originating from pUC backbone have generally been used for expression of heterologous proteins (15). Although, the pUC series of vectors were developed for cloning in the laboratory host strains of *E. coli* the vectors are compatible for use in *Salmonella.* Both *E. coli* and *Salmonella* belong to the family of *Enterobacteriaceae*, the group of gram-negative gut microbial flora that share significant genetic homology and plasmid compatibility (16, 17). The native *E. coli* plasmids with ColE1 origin of replication or vectors constructed for genetic manipulations bearing ColE1 derived replication origins are transmissible, stably maintained and well-partitioned during replication in *Salmonella* (18). However, there are no vectors available commercially that can constitutively express recombinant proteins under a strong promoter like P_{tac} or P_{trc} promoters.

This article details the construction of a vector for constitutive expression of heterologous genes in the Ty21a vaccine strain; and most likely in the other species of *Salmonella* and also other gram negative bacteria. A pBR322 based inducible expression vector (pProEx-HTb from Invitrogen) was chosen for modifications enabling constitutive expression of the cloned genes. We show that a simple reconstruction strategy of removing the *lac* I gene from the vector by employing the powerful tools of recombinant DNA technology- a hi-fidelity PCR followed by restriction enzyme cleavage and ligation enables constitutive expression of genes from the vector.

Materials and Methods

Plasmids and Bacterial hosts: The live-attenuated *S*. Typhi Ty21a vaccine strain and the constitutive expression vector for expression pFS14nsd was obtained from Dr. Denise Nardelli, CHUV- Laussane, Switzerland. The pProEx-HTb and E. coli strain Top10 were procured from Invitrogen® Corporation (USA).

Primers and PCR: Primers, *Mlu* I Forward primer, binding to the nucleotide sequence region 4750 to 4769 of pProEx-HTb (5' ATCTATACGCGTAATTAATGTGAGTTA GCGCG 3') and *Mlu* I Reverse primer, binding to the nucleotide sequence region, 3627 to 3646 of pProEx-HTb (5' AGTTCTACGCGTTGAATT GACTCTC TTCCG GG 3') were obtained from Bioserve™. Both primers were incorporated with *Mlu* I restriction site. The Polymerase chain reaction was performed with the HotStar Hi-fidelity polymerase kit ™ (Qiagen). The conditions set for PCR were as follows- a) Initial denaturation at 96ºC for 5 min. b) Thirty five cycles of denaturation at 96ºC for 30 sec; annealing at 60 ºC for 30sec and extension at 72 ºC for 4min. c) Final extension of primers at 72 ºC for 10min

Re-circularization of vector: The PCR amplified product was digested with the restriction enzyme, *Mlu* I (New England Biolabs, USA) according to the manufacturer's instruction. The digested product was then ligated with the T_4 DNA ligase (Roche Applied Sciences) following the procedure outlined by the manufacturer. Competent, *E. coli* Top 10 cells were transformed with the ligated product and then plated on Luria-Bertani Agar containing ampicillin (50µg/ml). Plasmid from the broth culture of a single colony of transformed Top 10 cells was extracted using Hispeed Plasmid Maxi Kit (Qiagen).

HPV genes: The codon-optimized HPV16 L1 gene in the pFS14nsd vector was obtained from Dr. Nardelli, CHUV, Lausanne. The codon-optimized HPV 16 E6 and E7 genes for expression in *Salmonella* Typhi were obtained as synthetic constructs cloned in a plasmid from GeneArt™, Germany.

Cloning: Plasmids containing HPV16 L1, E6 and E7 genes were restriction digested with Nco I and Hind III enzymes from New England Biolabs® Inc. for 3 hrs at 37ºC in the compatible buffer (Buffer-2) according to the manufacturer's instructions. The pSalEx vector was similarly digested with the corresponding Nco I and Hind III enzymes. Vector and gene ligation reactions

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were carried out with the Rapid Ligation Kit from Roche according to the manufacturer's instructions. Competent *E.coli* Top 10 cells were transformed with the ligated products and plasmids were purified either using the Qiaprep Plasmid Miniprep or the Hispeed Plasmid Maxi kit from Qiagen.

Expression: Competent *S. Typhi* Ty21a cells were electroporated with pSalEx clones of HPV16 L1S, E6 or E7. Cell lysates of the recombinant Ty21a thus obtained were screened for expression by western blotting procedure with the PVDF membranes according to the standard procedures involving electro-blotting (19). For qualitative comparison of expression, western blotting of the whole cell lysates of Ty21a recombinant containing the HPV16 L1S gene in the pFSnsd vector and the recombinant culture containing the gene in pSalEx was performed. Expression of HPV 16 L1S gene was probed with the commercial monoclonal antibody specific to protein, CAMVIR-1™ (Novus Biologicals). HPV 16 E6 and E7 genes were probed with monoclonal antibodies specific to the respective proteins procured from Santa Cruz Biotechnology, Inc.

Results and Discussion

Live-attenuated salmonella as an oral delivery vehicle of heterologous antigens from pathogens to confer immunological resistance to diseases is an attractive proposition. The approach requires that the heterologous antigen be expressed in adequate amounts to bring about an effective immune response to the antigen. An *in vivo* inducible system where the bacteria allows heterologous gene expression only after infection of the host cells is the ideal situation for evoking a robust immune response specific for the expressed antigen. However, construction of an inducible system is complex. It involves an intricate approach that is needed to strike the right balance between a strong, tightly regulated promoter system and the fitness of the bacteria. The hall-mark of attenuated vaccines is the poor survival ability inside the host cell thereby causing temporal limitation for optimal expression of the foreign antigen. A simpler alternative is to constitutively express the antigen under a strong promoter.

We envisaged that a constitutive vector suitable for expression in *Salmonella* can be constructed from an *E. coli* expression vector. The basis for such an idea originates from the use of plasmids based on ColE1 origin of replication in *Salmonella* for protein expression or as vectors for carrying heterologous DNA (15). The suitability of ColE1 origin for maintenance and replication of plasmids in *Salmonella* precludes the need for identification, isolation and selection of *Salmonella* specific plasmids. The published report on a pBR322 based pFS14nsd vector system with P_{tac} as the promoter for expression of a modified HPV16 L1 gene lends credence to the suitability of modified *E. coli* promoter systems for expression of proteins in *Salmonella* (10). Most *E. coli* inducible expression vector systems have been ingeniously adopted from the *in vivo* regulatory mechanism of the *lac* operon (20). Inhibition of expression before induction is mediated by the *lac* I or the modified *lac* I^q gene in these vectors, through its translated product the protein Lac I (or Lac I^q) (20). Therefore, the removal of *lac* I gene or abolishing its expression in an expression vector should logically render constitutive expression of the gene in *Salmonella*.

We chose the inducible expression vector, pProEx-HTb (currently discontinued by Invitrogen) for the modification into a constitutive vector. This vector is derived from the pBR322 vector. The hi-fidelity PCR with forward and reverse primers that flanked the 5' upstream and 3'down-stream of the *lac* I gene in the pProEx-HTb vector respectively amplified the ~ 3.8Kb length vector sequence minus the *lac* I as seen in Fig 1. The Mlu I site incorporated in both the primers enabled efficient ligation and re-circularization of the amplified product. The deletion of the *lac* I gene in the re-circularized pSalEx was verified by sequencing analysis (data not shown). The pSalEx vector retains all the

features of the pProEx including the multiple cloning site (MCS) and P_{trc} promoter upstream of the MCS. A conceptualized picture of the resulting vector from re-circularization of the PCR amplified product minus the *lac* I gene is illustrated in Fig 2.

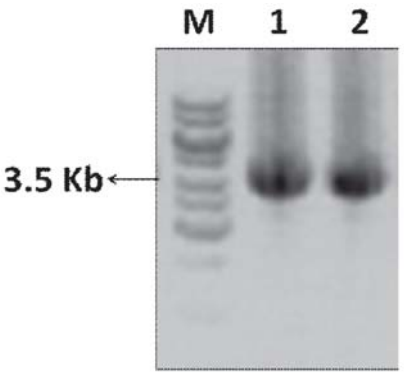

Fig. 1. Hi-fidelity PCR of pProEx-HTb with primers flanking the *lac* I gene. Lane M: Molecular weight DNA marker; Lane 1 and 2: Amplified pProEx-HTb vector region of ~3.8Kb devoid of *lac* I gene.

In order to test the suitability of the vector we chose the clinically relevant human papilloma virus genotype 16 (HPV 16) antigens, L1, E6 and E7 for expression in the vector. HPV infection causes cervical cancer in women and the genotype 16 of the virus is the most predominant type associated in cancer cases world-wide (21, 22). Recombinant Ty21a expressing the HPV16 L1 protein has been shown to elicit immune response in mice (10). Although its efficacy in humans would only be decided in planned clinical trials it holds promise as an oral vaccine and a cost-effective alternative to the virus like particle (VLP) based prophylactic vaccines (23, 24). We have attempted to correlate, although qualitatively, the expression of L1 from pSalEx to that seen in the candidate vaccine.

The HPV 16 LIS gene in the pFS14nsd vector is cloned in the *Nco* I and *Hin*d III sites and the sub-cloning of the gene in pSalEx was also carried out in the same sites. We sought to determine the ability of pSalEx to render expression of HPV16 L1S by the standard

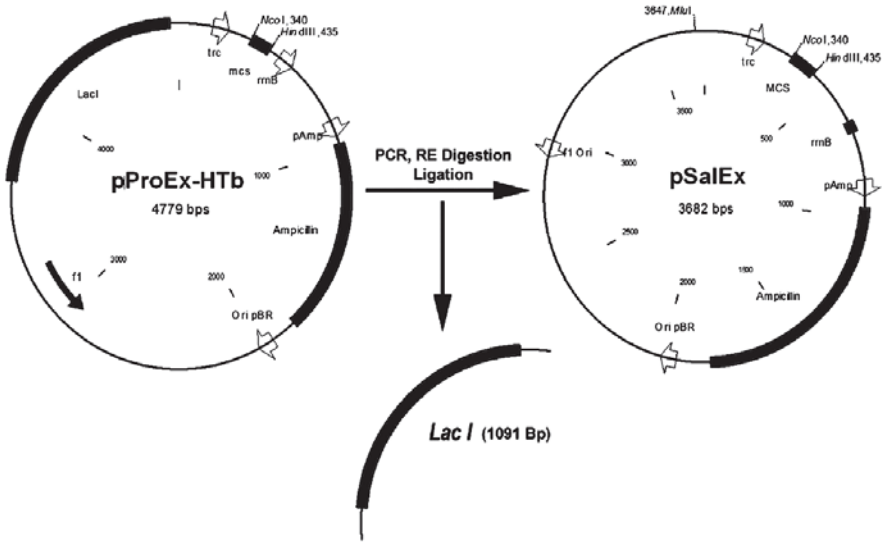

Fig. 2. Schematic illustration of the modification of pProEx-HTb to the constitutive expression vector pSalEx. *lac* I is removed by a PCR reaction with primers flanking the gene. Mlu I enzymatic digestion produces cohesive ends that enable efficient joining of the linear DNA. The T4 DNA ligase treatment covalently links the ends creating the re-circularized vector

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Western blotting procedure and also make a qualitative comparison of the expression to that seen from the P_{tac} promoter of the pFS14nsd vector (Fig. 3). However, quantitative analysis would help determine the effect of concentration on immune response to an antigen *in vivo*. The western blot profile of the HPV16L1 proteins (Fig. 3) is typical for the whole-cell bacterial lysates of L1 expressing recombinant Ty21a. Though the blot was probed with the monoclonal antibody, CAMVIR-1, specific for a linear epitope in the L1, the profile obtained is consistently of more than one band apart from the expected ~57.0 KDa (HPV16 L1 gene is 1515 bp in length). Fig. 3 shows a similar profile for pSalEx-HPV16L1 and pFS14nsd-HPV16L1 recombinant Ty21a indicating a similar levels of expression from the pSalEx vector to that of pFS14nsd.

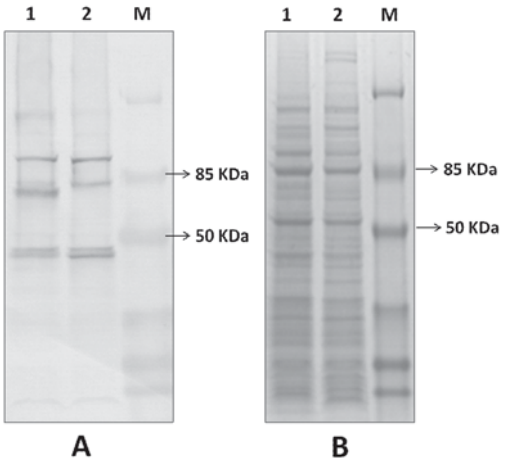

Fig. 3. Western blot of bacterial cell-lysates of HPV16 L1 recombinant *S.* Typhi Ty21a. A- Blot probed with HPV16 L1 specific monolclonal antibody, CAMVIR-1. B- Corresponding SDS-PAGE stained with coomassie brilliant blue. Lane 1: Bacterial cell-lysate from *Salmonella* Ty21a transformed with pFS14nsd cloned with the HPV 16 L1 gene; Lane 2: Bacterial cell-lysate from Salmonella Ty21a transformed with pSalEx cloned with the HPV 16 L1 gene; Lane M: Pre-stained Protein molecular weight marker.

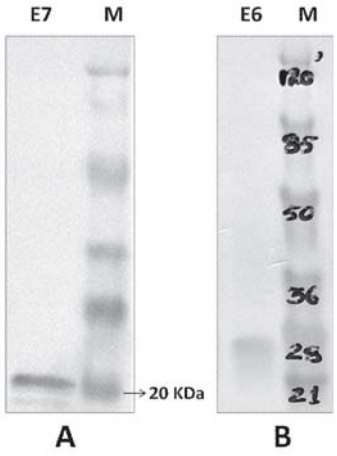

Fig. 4. Western blot of bacterial cell-lysates of HPV16 E6 and E7 recombinant *S.* Typhi Ty21a A- Blot probed with HPV16 E7 specific monolclonal antibody. B- Blot probed with HPV16 E6 specific monolclonal antibody. Lane M: Pre-stained Protein molecular weight marker; Lane E7: Bacterial cell-lysate from *Salmonella* Ty21a transformed with pSalEx cloned with the HPV 16 E7 gene; Lane E6: Bacterial cell-lysate from Salmonella Ty21a transformed with pSalEx cloned with the HPV 16 E6 gene

We cloned the clinically relevant, HPV 16 oncogenes E6 and E7 (25, 26) to further establish the strength of pSalEx as an expression vector. The western blot pictures (Fig. 4) confirm the expression. We need to mention here that pSalEx uses the synthetic *E.coli* promoter Ptrc for expression. Ptrc is identical in its nucleotide sequence to that of P_{tac} except that in P_{trc} the spacer sequences between the -35 and -10 element contains 15 bases compared to the 14 in P_{tac}. The relevance of the extended spacer on expression can only be unraveled by the quantitative determination of the expressed proteins in both pSalEx and pFSnsd.

The deletion of *lac* I does render the constitutive expression of antigens in pSalEx as confirmed by the results. But the vector requires further modification before being employed in the

development of vaccines for clinical use. The most important modification that needs to be carried out is to replace the selection marker in the vector. The ampicillin resistance gene, *bla*^{R} is the selection marker used in pSalEx. The kanamycin resistance gene, *kan*^{R} is the only antibiotic resistance gene approved for human use by the Food and Drug Administration (27). Therefore, *bla*^{R} needs to be replaced with either *kan*^{R} or a more acceptable selection marker such as genes that supplement nutritional auxotrophy. One of the characterized mutations in Ty21a is a point mutation in the isoleucine and valine biosynthetic gene, *ilv*D rendering it non-functional (8). A functional *ilv* D gene is therefore, a potential selection marker.

Conclusion

The results reiterate that vectors containing ColE1 derived origins of replication are adept for use in *Salmonella*. It also reiterates that the *E. coli* based synthetic promoters are suitable for expression in *Salmonella*. The use of *S*. Typhi Ty21a and other attenuated strains of *Salmonella* as bacterial vehicles for oral delivery of heterologous antigens are increasingly gaining ground. Researchers across the world are keen to develop suitable expression systems to optimize antigen delivery. This study strengthens the case for exploiting the large repertoire of *E. coli* expression vectors for expression of heterologous genes in *Salmonella*. The robust tools of recombinant DNA technology namely high-fidelity PCR and generation of synthetic DNA has given limitless options for vector modifications towards that end. Further modification to replace the ampicillin resistance gene *bla*^{R} with a clinically acceptable, selection marker would render the use of pSalEx vector in the development of recombinant Ty21a based oral vaccines.

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SEM and Elemental Studies of *Swertia chirayita*: A Critically Endangered Medicinal Herb of Temperate Himalayas

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Abstract

The plant *Swertia chirayita* is of immense medicinal importance. Due to its high demand in the pharmaceutical market and poor seed germination, the plant is categorized as critically endangered. In the present study, seed and pollen morphology has been investigated by light and scanning electron microscopy and elemental analysis has been performed. Seeds showed variation in seed shape and size. Seed coat pattern was reticulate-alveolate revealed through SEM studies. The pollen grains of *S. chirayita* studied were radially symmetrical and isopolar, oblate-spheroidal, inoperculate with reticulate ornamentation. Palynological and seed morphological characters are significant inidentification of a particular species. The microphotograph obtained with the help of scanning electron microscope (SEM) and weight percentage of specific elemental concentration obtained from energy dispersive X-ray spectroscopy attached to SEM are also reported. Elements play both curative and preventive roles against diseases. Roots samples showed higher concentration of Cl, K, Si, Al and Fe whereas concentration of Na, Ca, Cu, Zn, Mg were found high in leaves.

Keywords: Scanning electron microscopy, Elemental analysis, Gentianaceae, Palynology, *Swertia Chirayita.*

Introduction

Swertia chirayita (Roxb. ex Fleming) H. Karst (Gentianaceae) is a medicinal herb growing in the Himalayas from Kashmir to Bhutan and Khasi hills at altitude of 1000 - 3500 m(1). Some authors have described *S. chirayita* as an annual (2-3) and others as biennial or pluri-annual (4). The plant behaves differently due to climatic conditions or varying genotypes. Various species of genus *Swertia* reported are *S. paniculata, S. cordata, S. bimaculata, S. chirayita, S. nervosa, S. angustifolia.* Chen et al. (5) reported *S. changii* (Gentianaceae), as a new species from Southern Taiwan. *S. chirayita* grows well in sandy, loamy soil rich in carbon and humus. It has an erect, about 2–3 ft long stem, the middle portion is round, while the upper is four-angled. The stem is green in colour when young, but turns orange brown or purplish when matured. The leaves are lanceolate, and have five nerves varying in the length from eight to nine cm. The root is simple, tapering, stout and short. Flowers are small, stalked, and green-yellow and tinged with purple colour (2, 14) (Fig. 1). The entire plant is used medicinally. It has been used in the Ayurvedic system of medicines as a bitter stomachic, febrifuge, anthelmenthic, diuretic**,** antiepileptic and for certain type of mental disorders (6). Further, extracts of *S. chirayita* have been shown to possess antioxidative, antihepatotoxic and

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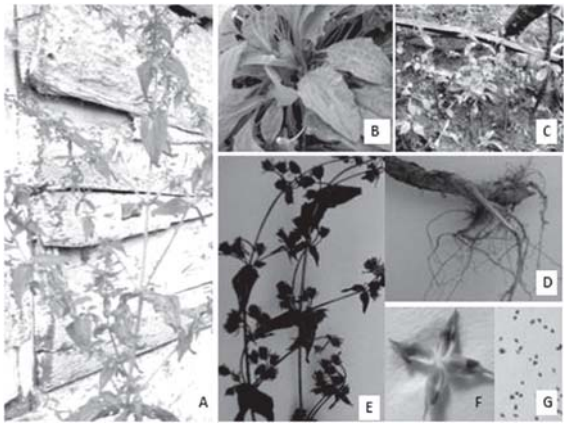

Fig. 1 *Swertia chirayita* A. 2 ft tall plant before flowering; B. Plant in vegetative phase; C.1 feet tall plant in wild habitat; D. Root of a mature two year old plant; E. Dry flowering twig showing matured capsules; F. Single tetramerous flower, G. Seeds

hypoglycemic, anti-inflammatory, antimalarial, anticarcinogenic, and antimicrobial activities (7). The major bioactives are xanthones, however, flavanoids, iridoids glycosides and triterpenoids are also active constituents of genus *Swertia* as reported (8). Herbal medicines such as Ayush-64, Diabecon (Himalayan herbal care), and Melicon Vointment (CadilaParmaceuticals) contain chiretta (colloquial name) extract in different proportions for its antipyretic, hypoglycemic, antifungal, and antibacterial properties (9-10-11).

Observations in many plant groups have shown that seed morphological characters are rather conservative which makes them taxonomically important (12-13). As study of seed structure of the genus *Swertia* has been neglected by previous workers, attention has been paid to the seed structure of *S. chirayita.* In Indian drug market, a number of adulterants have been detected along with the true "Chiretta ". The trade and economics of *S. chirayita* is affected by adulterants of the herb. *Andrographis paniculata* (commonly known as green chirayita), *Swertia alata* Royle.,*S. angustifolia* Buch.-Ham., *S. bimaculata* Hook. f. and Thoms., *S. ciliate* G. Don, *S. densifolia* Greisb. are adulterants found along with true chirayita. The true chirayita can be distinguished from other substitutes and adulterants by its intense bitterness, brownish-purple stem (dark colour), continuous yellowish pith and petals with double nectaries (14). In genus *Swertia,* morphological, biochemical (15-16), isozyme markers (17), AFLP marker (18) has been used for demarcating different species. Such studies highlighted the importance of having diagnostic keys for evaluating the authenticity of the available material. In this context, SEM studies will help in identifying and documenting authentic samples. The present study was undertaken with the following objectives: (a) to study the seed shape, size and its seed coat pattern (b) anther and pollen study (c) elemental analysis of plant sample in relation to its immense medicinal potential.

Materials and Methods

Scanning electron microscopy (SEM) studies: Authenticated seed samples were provided to us by Ms. Pramila Choudhary, Director, Organoindia Organisation, Tung, Darjeeling, India. Only matured capsules were selected and seeds were separated for investigation. The dry seeds were cleaned and examined by light microscope (LM) to study the shape and colour of seed. For SEM investigations, the seeds were dried and fixed to specimen stubs with an adhesive and placed on the revolving discs of Joel fine coat ion sputter (JEOL, JFC 1600),where each seed was uniformly coated with 20-30 nm thick gold. These specimen stubs were then fixed to the specimen holder of Scanning Electron Microscope (JEOL JSM 6390LV) maintained at accelerating potential voltage of 20 KV and photomicrographs were taken at different magnifications (seed in whole mount with ×= 50 and Seed scan with ×=2200). The terms used for describing the seed coat patterns have been adopted according to Murley (19) and Koul et al. (20). For palynological studies, pollen grains were coated with gold in sputter-coater and analysed using Scanning Electron Microscope. The pollen terminology follows Brochmann (21).

Elemental analysis: The plant material was collected from Tung, Darjeeling (WB) at 2000m.of altitude.The leaf, stem and roots were carefully separated from the plant. The cleaned plant parts were dried in shade in a clean environment to avoid the contamination. The photomicrographs of these samples were taken using SEM. Elemental analysis to identify the weight percentage of various elements present in the samples were done using OXFORD DCL7673 energy dispersive X-ray (EDX).

Results and Discussion

SEM studies: As an aid to identify plant materials by botanists, drug plant buyers, seed scientists and ecologists, seed structure has been of immense importance. Usually, the external features of seeds are used for identification. Seeds of *S. chirayita* showed wide variation in shapes and sizes. In the present study, the shapes of seeds showed wide variation from ovoid to spherical, subspherical, ellipsoidal, round, rectangular and sometimes laterally compressed. LM studies showed colour of the seeds varying from light brown to brownish black (Fig. 1G, 2). The size of seeds also varied from 0.21mm × 0.22mm to 0.28mm ×0.65mm. (Fig. 3, 4).The seeds studied were not winged. The seed coat pattern as investigated through SEM studies revealed reticulate-alveolate pattern. The periclinal walls were smooth with deeply concave cells and anticlinal walls elevated and smooth. Epicuticular waxes were present on the reticulate excavations of cuticle. The capsules were lanceolate and possess numerous seeds. The number of seeds in the capsules of *S. chirayita* was found to be 100-110. Investigations revealed micropylar dimensions 0.09mm ×0.03mm.The number of seeds in the capsules varied from 4 in *S. macrosperma* to 300 in *S. alata* (22). They reported winged seeds found in *S. hookeri, S. alternifolia*, whereas no wings are present in *S. ramosa* and *S. petiolata* seeds. In *S.changii*,

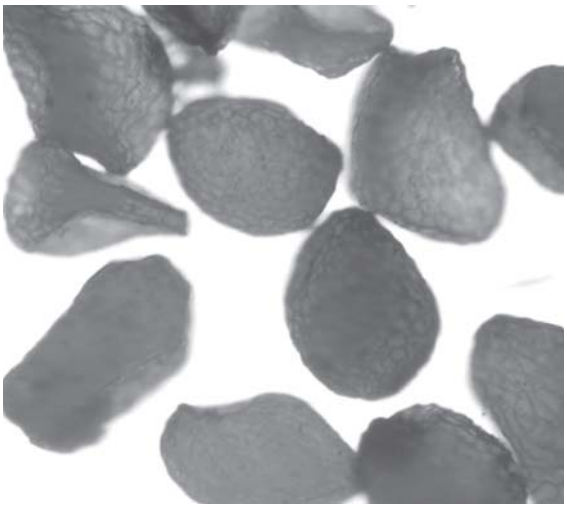

Fig. 2. Seeds of *Swertia chirayita* as seen under LM

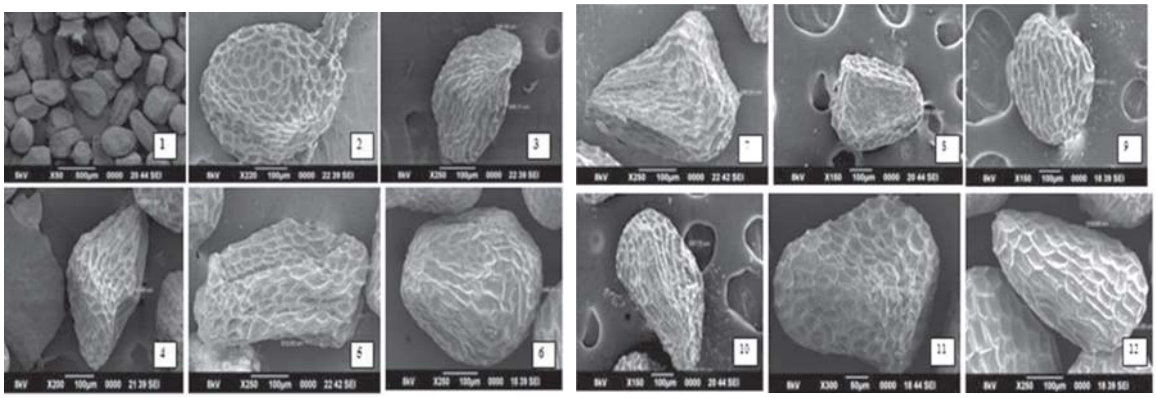

Fig. 3. (1-12) Scanning electron micrographs of *Swertia chirayita* seeds showing varied seed shape; seed coat pattern is reticulate- alveolate

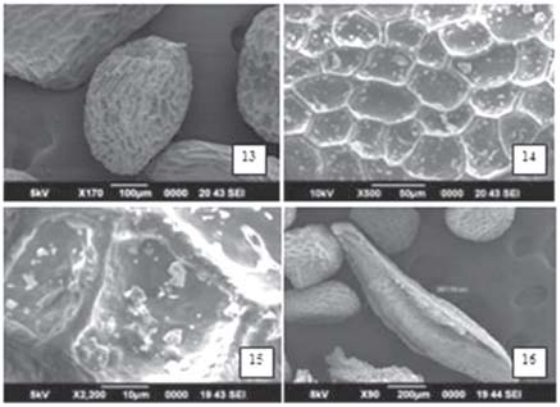

Fig.4. (13) Seed showing dimensions of micropylar region; (14, 15) Reticulate-alveolate seed coat pattern; (16) Capsule of *S. chirayita*.

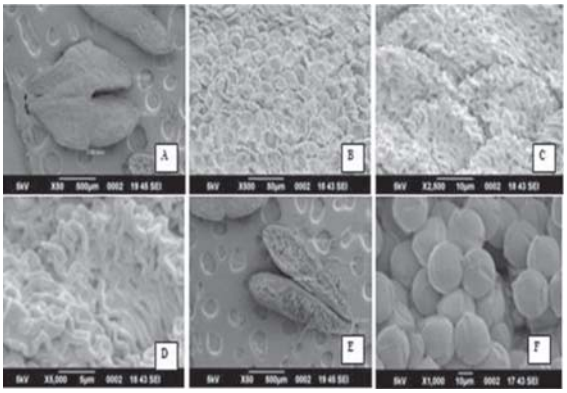

Fig. 5. A.Dorsal view of anther of flower of *S. chirayita* showing dimensions; B. honeycomb surface pattern of anther; C. Enlarged view of surface pattern; D. single compartment enlarged view showing wavy ridges; E. anther lobes containing mass of pollen; F. pollen grains of *S. chirayita*

Chen et al. (5) reported that the seed coat was echinate showing smaller protrusions of the epidermal cells on the seed coat.

Pollen morphology: Anther and pollen morphology has been investigated through scanning electron microscopy in *S. chirayita*. The anther lobes showed little variation in dimensions (Fig.5). The outer wall of anther lobes showed honey comb like pattern with compartments showing wavy ridges. The pollen grains (Fig.6) were observed to be radially symmetrical and isopolar. The shape was spheroidal. The pollen grains were tricolpate. The exine ornamentation was reticulate. Colpi were generally narrow and deeply sunken in the exine. Aperture length was more than 4/5 of polar axis. Colpus margin was regular and membrane colpi granulate. In *S. bimaculata* and *S. perenis* exine was reported to be striate-perforated and striate-perforate microreticulate (23). In *S.changii*, pollen grains found were tricolporate, isopolar, spheroical to prolatespheroical in equatorial view and semiangular in polar view, with long colpi, ends acuminate, exine regulate, with 1-2 ìm striae (5).

Elemental analysis: Nature has provided us herbal medicines under different climatic conditions and all basic principles pertaining to human therapy have been derived from medicinal plants and herbs **(**24). Herbal medicines are widely used in many countries; only small number of plant species had been scientifically validated to support their use worldwide. Elements play both curative and preventive roles against diseases. The majority of trace elements act as catalysts in a variety of enzyme system functions

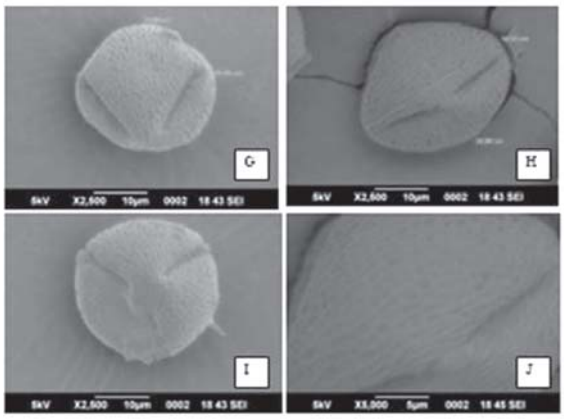

Fig. 6. G. Pollen of *S. chirayita* showing polar view, dimensions shown; H. pollen showing equilateral view, with dimensions; I. Pollen in polar view; J. Equilateral view of pollen showing granular aperture

(25). The elemental composition and concentration of *S. chirayita* were investigated by energy dispersive X-ray fluorescence.

Table 1. Elemental composition of leaves, stem and roots of *Swertia chirayita*

Elements (Wt %)	Leaf (Wt %)	Stem (Wt %)	Root
Mg	0.60	0.18	0.32
Al	0.12	0.37	1.44
Si	0.54	0.49	1.47
Cl	0.56	0.33	2.09
K	0.88	1.83	1.89
Ca	0.78	0.37	0.45
Cu	0.64	0.60	0.60
Na	_	0.18	1.37
Zn	0.61	0.47	0.60
Fe	-	0.19	1.05

Table 1 revealed significant presence of silica, calcium, chloride, magnesium, aluminum, sodium, potassium, zinc, copper and iron in leaves, stem and roots of *S. chirayita.* The distribution of these elements was not homogenous. Concentration of elements was found in the order Cl>K>Si>Al>Na>Fe>Ca> Cu>Zn>Mg. Major concentrations were found in roots than in leaves. Presence of eight trace elements had been studied in *S.davidii* Franch (26), six in *S.yunnanensis* (27) by atomic absorption spectrometry and Pb, Zn, Cu, and Ni have been analyzed in a *S. densiflora* by optical emission spectroscopy (28). Nine elements (Zn, Cu, Fe, Co, Mn, Na, K, Ca, Li) in *S. chirayita* and *S. speciosa* were analyzed by atomic absorption spectrometry (29; 25). Na and K are electrolytes and play an important role in maintaining acid base balance in body and blood pressure. Maximum concentration of Na was found in roots than stems and K also in roots followed with stem and leaves. Calcium is an important macronutrient and believed to possess the ability to prevent precancerous cell morpho-differentiation into malignancy by binding to cancer promoting fats thus inhibiting their ability to initiate cancerous growth. It is also an important regulator of many cellular mitotic activities (30). It is the main component of bones and teeth. It also helps in the process of coagulation, regulation of heart beat, cellular permeability, muscular contraction, transmission of the nerve impulses and enzymatic activity. Sodium, potassium and calcium play an important role in the electrophysiology of cardiac tissue. Calcium ions increase the force of contraction of the heart. Joshi and Dhawn (14) reviewed *S. chirayita* as cardio-stimulant. The combination of calcium and silica may be associated with bone repairs and could be useful in formation of collagen. Magnesium, aluminium, potassium and copperare utilized as trace elements in enzymatic and other metabolic functions. Zinc and iron are important for wound healing and blood formation. The results of the present study provide justification for the usage of this medicinal plant in the treatment of diabetes mellitus (DM) since it is found to contain appreciable amounts of the elements like K, Ca, Cu, and Zn, which are responsible for potentiating insulin action. Our results are in concurrence with the results obtained by Naga Raju (31) and group, who justified that the analyzed medicinal plants could be considered as potential source for providing a reasonable amount of the required elements for the patients of DM. Moreover, these results could be used to set new standards for prescribing the dosage of the herbal drugs prepared from these plant materials. Zn has been well known to be an important trace element in diabetes as a cofactorfor insulin. Patients with diabetes mellitus tend to have low serum zinc and increased urinary excretion. Zn has a low order of toxicity as compared with most of the other trace elements and it enhances the effectiveness of insulin secretion (32). The deficiency of iron causes anemia especially during the period of pregnancy. The Ayurvedic medicine “chiretta” is prepared from *Swertia* species containing high concentration of iron

which could reduce iron deficiency to prevent anemia. Hence it is concluded that studies regarding elemental analysis in herbal preparations are useful for mankind as they play significant role in combating a variety of human diseases.

Conclusion

Seed morphology studies provide a number of characters potentially useful for species identification and phylogenetic inference (33-34-35). The seeds of *Swertia chirayita* showed a great variation in size and shape. SEM studies of seed and pollen would be helpful for the identification of the species. Elemental studies of roots samples showed higher concentration of Cl, K, Si, Al and Fe but Na, Ca, Cu, Zn, Mg were found maximum in leaves. Moreover, these results could be used to set new standards for prescribing the dosage of the herbal drugs prepared from these plant materials.

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NEWS ITEM

Take a serious look at medical curriculum: Prime Minister Manmohan Singh

Speaking at the third convocation of Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Prime Minister Manmohan Singh emphasised the need to take a serious look at the curriculum for medical education so that doctors are trained to look at health in a holistic manner that goes beyond a narrow clinical and technology-driven approach. He said that medical education should be reconfigured to produce technically competent, socially sensitive, ethically correct and ready to serve health professional, who can respond to the diverse demands of India's health needs. "Students training to be doctors have to be prepared to work with local communities and in our villages. They should be sensitized to the social determinants of health and be as willing to contribute to preventive healthcare and its management as the more lucrative curative systems," he said. Stressing that education of health professionals must be transformed in precept and practice, the PM said interdisciplinary learning and health system connectivity should become the hallmarks of contemporary medical education. Concerned over the quality of medical education, the PM called for establishing a credible regulatory and institutional mechanism to help develop standards in medical education.

Exclusivity in education not the answer: Kapil Sibal, Minister of Human Resource Development, India

Speaking at the India Today Aspire Education Summit 2012, Union HRD minister Kapil Sibal made a strong case for inclusivity in education and said true democratisation of a nation began in the classroom. Citing statistics, Sibal said one in every 100 children is a genius, so it would be a shame if that genius never got a chance to go to school. While in school, a child must not merely be a recipient of knowledge but allowed to be a creator too. The child must be free to exercise his spirit of enquiry, given choices in the classroom so that he can go on to exercise them later in life. He said that this would lead to a society which does not look down upon those who work with their hands or listen to their conscience. Sibal said education was the only way India could fulfil its economic dreams. To join the ranks of the developed nations, the minister said the country needed a critical mass of people going to school, followed by university, who are the creators of wealth.

Keep high aims and defeat problems: Abdul Kalam

At the diamond jubilee celebrations of Sri Shanmukhananda Fine Arts and Sangeetha Sabha, Former President APJ Abdul Kalam advised the youth to keep high aims, regularly update their knowledge and succeed by defeating problems in life. While explaining the four tools to fight the problems in life, Kalam said that a person should have great aim, should continuously engage himself in acquiring knowledge - by reading great books, hard work and perseverance. He motivated youth by advising them to defeat the problem and succeed as nobody succeeded without problems and to become the captain of the problem and succeed. Kalam said that youth have to fight the hardest battle, which any human being can ever imagine to fight and not to stop fighting until the destiny is arrived. He added that youth should be ready to contribute to the national development for a transparent and corruption-free society.

Institute on research and innovation to come up in India: Kapil Sibal

A first-of-its kind institute in the Pacific region, dedicated to research and innovation and regional networking, will be set up in the country as a UNESCO category one centre. To be named as Mahatma Gandhi Institute Of Education for Peace and Sustainable Development, the institute would be inspired by Gandhi's vision of peace and sustainability. An agreement in this regard was signed between HRD Minister Kapil Sibal and Director General of UNESCO Irina Bokova in Paris. HRD Minister Kapil Sibal said that the institute's core activity will lie in research and capacity building. It will encourage knowledge exchange, regional networking and catalyse innovation by helping to design and test new approaches to education, he added. "The institute comes at the right time, a time when the world is debating the contours of the century ahead. As the challenges of the 21st century are qualitatively different from the challenges of the 20th century, global understanding and education would assist in appreciating the impact of these challenges on peace and its relation to sustainable development," Sibal said after signing the agreement.

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SCIENTIFIC NEWS

First Complete Computer Model of an Organism being produced

Stanford researchers reported that in a breakthrough effort for computational biology, the world's first complete computer model of an organism Mycoplasma genitalium, the world's smallest free-living bacterium has been completed. Mycoplasma genitalium is a humble parasitic bacterium known mainly for showing up uninvited in human urogenital and respiratory tracts. The researchers modeled individual biological processes as 28 separate "modules," each governed by its own algorithm. These modules then communicated to each other after every time step, making for a unified whole that closely matched M. genitalium's real-world behavior. The program also allowed the researchers to address aspects of cell behavior that emerge from vast numbers of interacting factors. This achievement demonstrates a transforming approach to answering questions about fundamental biological processes. Not only does the model allow researchers to address questions that aren't practical to examine otherwise, it represents a stepping-stone toward the use of computer-aided design in bioengineering and medicine. . Computational models like that of M. genitalium could bring rational design to biology — allowing not only for computer-guided experimental regimes, but also for the wholesale creation of new microorganisms. Comprehensive computer models of entire cells have the potential to advance our understanding of cellular function and, ultimately, to inform new approaches for the diagnosis and treatment of disease.Once similar models have been devised for more experimentally tractable organisms, bacteria or yeast specifically designed to mass-produce pharmaceuticals. Bio-CAD could also lead to enticing medical advances — especially in the field of personalized medicine.

N.Vijaya Sree

Mineral-rich pearls to aid in fighting cancer

Pearls rich in essential minerals can help treat killer diseases like cancer. In a series of experiments by Ajai Kumar Sonkar at the Pearl Aquaculture Research Foundation in Port Blair, pearls produced through special culture technique have been found to contain traces of several metals and minerals such as zinc, copper, magnesium, iron, calcium, sodium and potassium which are known to have major health benefits and are essential for various body functions such as metabolism, growth and immunity. Of them, zinc has been found to be playing a major role in preventing fatal diseases like cancer. A study, published recently in the British Journal of Cancer, has also established zinc's anti-tumour role that prevents the growth of cancer cells. Sonkar produced pearls from four different species of pearl oysters. The bio availability of zinc in the pearls can be exploited to help treat several diseases. Scientific analysis of pearl powder samples was carried at Indian Council of Agricultural Research's Central Institute of Fisheries Technology in Cochin, which established the pearls do contain all the mentioned metals and minerals. In the pearl culturing operation, one to three-years-old oysters undergo surgical implantation, known as seeding, in which mantle issue is taken from the donor oyster and grafted in the recipient oyster along with the nucleus. Then these oysters are kept in laboratory condition for healing, after which they are transferred to the sea placed in the cages where they remain six months to two year for pearl formation. The oyster can produce more than one pearl in its lifetime by taking good care of it, including regular cleaning of the outer shell to remove seaweed. Other studies have also found that zinc deficiency in the body causes delayed healing of wounds. It is also found to play a

leading role in weight loss, help decrease the severity and duration of cold and several other illnesses.

P. Udaya Sri

Psoriasis and Wound Healing Gene Identified

A gene has been identified that regulates keratinocyte proliferation and differentiation after skin injury and, when overexpressed, can induce the autoimmune skin disorder psoriasis. Investigators at the University of California, San Diego (USA) analyzed skin biopsies of patients with and without psoriasis, as well as the skin of mice with psoriasis and with wounds on their backs. They reported that regenerating islet-derived protein 3-alpha (REG3A) was highly expressed in keratinocytes during psoriasis and wound repair and in induced psoriatic skin lesions in mice. This gene encodes a pancreatic secretory protein that may be involved in cell proliferation or differentiation. The enhanced expression of this gene has been observed during pancreatic inflammation and liver carcinogenesis. The expression of REG3A by keratinocytes was shown to be induced by interleukin-17 (IL-17) via activation of the keratinocyte-encoded IL-17 receptor A (IL-17RA). This activity acted to inhibit terminal differentiation and increased cell proliferation by binding to exostosin-like 3 (EXTL3) protein followed by activation of phosphatidylinositol 3 kinase (PI3K) and the kinase Akt (protein kinase B). The discovery of REG3A's dual roles provides a new target for different therapies. A drug that inhibits the expression of REG3A could represent a more targeted way to treat psoriasis without the systemic immunosuppression problems of current treatments. Conversely, a drug that stimulates or mimics REG3A could boost cell growth and improve wound healing.

S. Jeevan Amos

Loss of Tiny Liver Molecule Might Lead to Liver Cancer

A new study showed that loss of a small RNA molecule in liver cells might cause liver cancer and that restoring the molecule might slow tumor growth and offer a new way to treat the disease. The animal study led by researchers at the Ohio State University Comprehensive Cancer Center examined what happens when liver cells lack a molecule called microRNA-122 (miR-122). They found that when the molecule is missing, the liver develops fat deposits, inflammation and tumors that resemble hepatocellular carcinoma (HCC), the most common form of liver cancer. When the researchers artificially restored miR-122 to nearly normal levels by delivering the miR-122 gene into liver cells, it dramatically reduced the size and number of tumors, with tumors making up 8 percent on average of liver surface area in treated animals versus 40 percent in control animals. These findings reveal that miR-122 has a critical tumor-suppressor role in the healthy liver, and they highlight the possible therapeutic value of miR-122 replacement for some patients with liver cancer. These findings also demonstrate what happens when miR-122 is lost in liver cells, and this might help improve the safety of new drugs that treat hepatitis C virus infection by blocking miR-122.

D. Siva Mallika

EDUCATION

PhD/Post Doctoral Programs

Admission to Ph.D and Post Doctoral positions in RNA structural bioinformatics / crystallography, International Institute of Molecular and Cell Biology (IIMCB), Warsaw, Poland: Applications are invited for a PhD and posdoctoral positions in a project aiming at structural characterization of RNAs and protein-RNA complexes at Bujnicki laboratory in the International Institute of Molecular and Cell Biology (IIMCB), Warsaw, Poland (http://iimcb.gov.pl). For a postdoctoral researcher (Starting January 1st, 2013, contract for 3 years), candidate familiar with molecular biology and with extensive experience in using various bioinformatics tools, in particular for comparative sequence analyses and structure prediction is required. Experience in structural / evolutionary bioinformatics, sequence analyses, database searches, phylogenetic studies, macromolecular structure prediction is desirable. Applicants for the postdoctoral position must also hold PhD in computational, natural of life sciences, have experience in bioinformatics documented by publications, know Linux and have at least intermediate skills in programming in Python, demonstrate fluency in English (written and spoken). For a PhD position (Starting October 1st, 2012 funded for 4 years) candidate with background in experimental structural biology and/or biochemistry is required. Applicants for the PhD position must have a MSc degree and be eligible for admission to a PhD programme under supervision of prof. Bujnicki, have experience in experimental analyses of proteins & DNA/RNA, demonstrate fluency in English (written and spoken). Applications for either position must contain Curriculum vitae, List of publications and/or major achievements, Motivation for applying, Contact details of at least two persons for references including one from the previous supervisor. Applications are collected only by email to:

employment@genesilico.pl until August 31st, 2012. Selected candidates will be contacted in the first week of September. Interviews will be held on September 10th. For more information visit the website: http://iimcb.genesilico.pl. For further details contact: Head of the Laboratory, Janusz M. Bujnicki, PhD, DSc, Professor of Biological Sciences, Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology in Warsaw, ul. Ks.Trojdena 4, 02-109 Warsaw,Poland. Tel: (+48-22) 597-07-50. Email: iamb@genesilico.pl.

Admission to Postdoc Position in systems biology with applications in prostate cancer, Karolinska Institutet, Sweden: Applications are invited for a two-years postdoctoral research position in Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Sweden, to characterize prostate cancer progression, aggressiveness and prognosis by integrating genetic and transcriptomics deep sequencing data with clinical information using a large population based prostate cancer cohort. The applicant is expected to work with high-level data analysis and modeling in the field of medical systems biology using high quality in-house produced data. The results from computational analyses will be validated with independent experiments. The applicant should have a PhD degree in computer science, applied mathematics, bioinformatics, systems biology, or related discipline. Ideally, applicant has strong methodological expertise (e.g., graph theory, probability theory, machine learning), good programming and communication skills, and is interested in developing and applying novel computational methods in cancer research. The application should contain complete curriculum vitae, including date of the thesis defence, title of the thesis, previous academic positions, academic title, current position, academic distinctions, and committee work, a complete list of publications, and a brief statement of research interest documents in English or Swedish. Eligible candidates can send applications to Karolinska Institutet, Department of Medical Epidemiology and Biostatistics, PO Box 281, SE-171 77, Stockholm, SWEDEN. Telephone No: +46-8-524 85000. For further details visit the website: www.ki.se/meb.

OPPORTUNITIES

Indian Agricultural Research Institute, Regional Station, Indore–452 001 (M.P.), India. Applications are invited from eligible candidates for one Senior Research Fellow (SRF) position to work in a research project entitled “Biofortication of wheat for micronutrients through conventional & molecular approaches” sponsored by the Department of Biotechnology, New Delhi, India. Candidates with M.Sc. degree in Plant Breeding and/or Genetics/Agricultural Botany/Life Sciences/Biochemistry with minimum two years research experience are eligible. Experience of working with crop plants and Basic computer skills are desirable. The interview of eligible candidates for the above post is held on August 13, 2012 at 11:00 A.M. at IARI-Regional Station, Residency Area (Near College of Agriculture), Indore–452 001 (M.P.), India. Eligible candidates fulfilling all the requirements should bring their application in the format giving full details of academic records and experience along with attested photocopies as well as original copies of relevant documents along with one passport size photograph.

Tea Research Association, Jorhat-785008, Assam, India. Applications are invited from eligible candidates for position of Head, Plant Physiology and Breeding Department (Scientist F) with pay scale of Rs 37,000–67,000, Grade Pay Rs 8900, PB-4 with other facilities like PF, Medical, semi furnished accommodation, regional allowance, etc. as per Rules. Candidates with Ph.D. in Plant Physiology/ Breeding/Taxonomy with minimum 15 years experience in plant research preferably in tea and other plantation crop/horticulture/forestry, out of which at least five years in senior supervisory position are eligible. Experience in research in reputed institutes and quality published work in high impact factor journals demonstrating exceptional research abilities are desirable. Eligible candidates may submit their application as per specific format available in the website www.tocklai.net along with certificate copies, etc. to The Director, Tea Research Association, Tocklai Experimental Station, Jorhat 785 008, Assam, India, Tel: 91-0376-2360467, 0376-2360972, Fax: 91-0376-23600974. E-mail: tratjorh@rediffmail.com or info@tocklai.net on or before 31 July 2012.

University of Mysore, Mysore - 570 006, India. Applications are invited from eligible candidates for the posts of four Research Associates and ten Junior Research Fellows, to work in an inter-disciplinary research project in the area of Nanotechnology entitled “Processing, Characterization and Applications of Advanced Functional Materials” sponsored by the University Grants Commission, New Delhi, under the University with Potential for Excellence Program for a period of five years. The interested candidates can

send their applications to Prof. K. Byrappa, Coordinator, UPE, University of Mysore, NCHS Building, IQAC Office, Opposite to EMMRC, Manasagangothri, Mysore 570 006, Karnataka, India on or before 30 July 2012. For more details contact: kbyrappa@gmail.com.

Acharya Nagarjuna University, Andhra Pradesh, India. Applications are invited from eligible candidates for the Position of Junior Research Fellow (JRF) for 3 years to work on a project entitled "Biodiversity of Indian caves with special reference to Copepoda and Bathynellacea (Crustacea)" funded by DST, New Delhi. Candidates with M.Sc. in zoology with atleast 60% marks are eligible. CSIR/NET qualified candidates are desirable. Pay scale: Rs 16,000/- along with HRA. The applications by mail/email should reach to Dr.Y.Ranga Reddy, Principal Investigator, Department of Zoology, Acharya Nagarjuna University, Nagarjunanagar – 522510, Andhra Pradesh, India. E.mail: yrangareddy@yahoo.com.

Indian Agricultural Research Institute, New Delhi- 110012, India. Applications are invited from eligible candidates for one Senior Research Fellow (SRF) position to work in a research project entitled "Molecular Characterization of Phytoplasmas Associated with Sugarcane Crops in India" sponsored by the Department of Science and Technology, New Delhi, India. Candidates with M. Sc. in Biotechnology/ Plant Pathology/ Life Sciences/Molecular biology; NET/ GATE are eligible. Research experience in basic techniques in Plant Pathology, Insect transmission and Molecular biology (knowledge of PCR, cloning, sequence comparison etc) is desirable. Pay scale: NET/ GATE + 2 years experience: Rs. 18000 + HRA or NET/ GATE without experience: Rs. 16000 + HRA. Application should be submitted to Dr G.P. Rao, Principal Investigator, DST project, Principal Scientist, Division of mycology & Plant Pathology, Indian Agricultural research institute, New Delhi - 110012 (e mail: gprao_gor@rediffmail.com). Eligible candidates can attend for the interview on August 24, 2012 at 10:00 A.M. in the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi- 110012, India.

SEMINARS/WORKSHOPS/CONFERENCES

BIOFEST-2012 International Bio Conference and Event: Biofest-2012 International Bio Conference and Event with the main theme of "Exploit current research for harnessing the field of Life sciences" was going to held on December 12-13, 2012 at Leonia International Centre for Exhibitions & Conventions, Hyderabad, India organized by Bright International Conferences & Events Organization, Hyderabad, India. Abstract can be submitted online through website: http://www.brightice.org/abstract-submission.php on or before August 15th, 2012. For further details contact: Conference Secreteriat-Biofest 2012, Bright International Conferences & Events, # 14-155,Shakthi sai nagar, Mallapur, Hyderabad-500076, India. Email: biofest2012@brightice.org, info.brightice@gmail.com,info@brightice.org. Phone: +91-40-64540825.

National Conference on Aquatic Animal Health and Management (NCAAHM - 2012): A National conference on Aquatic Animal Health and Management (NCAAHM - 2012) was going to held on September 14 - 15th, 2012 at Annamalai University organized by Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608 502, Tamil Nadu, India. Abstract can be submitted online through E-mail: animalhealth2012@gmail.com or sravicas@gmail.com on or before August 20th, 2012. Special awards and testimonials will be presented to the best presentations in both oral and poster categories. For further details contact: Dr. S. Ravichandran, Organising Secretary, NCAAHM – 2012, Parasitology labCAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608 502, TamilNadu. India. E-mail to: sravicas@gmail.com, animalhealth2012@gmail.com. Phone No: 9443909137, 04144 243233.

National Conference on Nanomaterials - (NCN-2012): A National Conference on Nanomaterials - (NCN-2012) was going to held on December 3-4, 2012 at Karunya University, Coimbatore, Tamilnadu, India organized by Department of Physics, School of Science & Humanities, Karunya University, Coimbatore – 641 114, Tamilnadu, India. Abstract can be submitted online through E-mail: ncn2012@karunya.edu on or before October 15, 2012. For further details contact: Dr.A.Kingson Solomon Jeevaraj, Organizing Secretary, NCN-2012, Department of Physics, School of Science & Humanities, Karunya University, Coimbatore – 641 114, India. E-mail: ncn2012@karunya.edu. Phone: +91 9842466973.

AP Science Congress: The 5th A.P Science Congress with a focal theme of Innovations In Science, Technology And Mathematics (ISTAM) was go-

ing to held on 14th- 16th November, 2012 at Acharya Nagarjuna University, Nagarjunanagar, Guntur, Andhra Pradesh, India organized by Acharya Nagarjuna University and Andhra Pradesh Akademi Of Sciences (APAS), Andhra Pradesh, India. Abstract can be submitted online through Email: 2012apsc@gmail.com, apas1963@yahoo.co.in. on or before october 25th, 2012. For further details contact: Prof.K.R.S. Sambasiva Rao, Organizing Secretary, APSC-2012, Department of Biotechnology, Acharya Nagarjuna University, Nagarjunanagar – 522 510, Guntur, A.P, India.

Workshop on Molecular Biotechnology and Bioinformatics, Pune, India: A 5-day workshop on Molecular Biotechnology and Bioinformatics was going to held on 27th – 31st August 2012 at International Center for Stem Cells, Cancer and Biotechnology (ICSCCB), Pune, India organized by International Center for Stem Cells, Cancer and Biotechnology (ICSCCB), Director, ICSCCB, R.H. No. 2, Ujwal Regalia, Near Prabhavee Tech Park, Baner Road, Pune – 411 045, India. UG/PG/PhD students, faculty, scientists as well as people working in industry in the field of Biotechnology, Bioinformatics, Life Sciences, Medical Sciences, Pharmaceutical Sciences, Chemical Sciences and related subject areas are eligible. Interested applicants can download the Workshop Registration Form and send it along with a Draft of Rs. 6000/- in favor of 'Agamya Biotech Private Limited' payable at 'Pune' to the address given on the form. For further details contact Prof. Dr. Sheo Mohan Singh, MSc(UK), PhD(Germany), PDF(USA,UK), Director, ICSCCB, Pune, India. Email: info@icsccb.org or icsccb2012@gmail.com. Tel No: +91-9545089202.

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