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Production and Characterization of Bioactive Peptides by Purified Protease Isolated From *Bacillus* Sp. Mtcc 9558

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Abstract

Enzymes are extensively studied for its use in production of bioactive peptides, and due to their great potential they find enomorus commercial applications. The study is use of purified protease in protein hydrolysis for bioactive peptide production and its applications. The protein rich natural sources such as spirulina, yeast, casein, soy protein and β-lactoglobulin were selected and were prepared as protein substrates of protease. Upon reaction, the peptides samples were analysed for their antioxidant, antimicrobial and antithrombotic properties. Antioxidant activities and potential of the protease from *Bacillus* sp. to generate antibacterial peptides from different natural protein sources was also studied. The results of antithrombotic activity of bioactive peptides from different protein sources indicated inhibitory activities on the thrombin-catalyzed coagulation of fibrinogen. The potent inhibitory effects were observed for casein peptides. The peptides were termed bioactive peptides as they exhibited the above mentioned activities. The bioactive peptides were also characterized and profile of its amino acid content was determined. The presence and characterization of bioactive peptides were confirmed by Tricine-SDS-PAGE and LC-MS-MS analysis. The results were analyzed for the presence of essential amino acids. The results indicated that the protein peptides have potential applications as food supplements. As the protease hydrolyzed peptides containing hydrophobic amino acids, are much useful in reducing the bitterness by removing these amino acids. Therefore, *Bacillus* sp. protease may have the scope to eliminate the bitter taste by selective hydrolysis of bitter peptides and may offer possibilities for debittering of protein hydrolysates and utilization of non-conventional food sources.

Keywords Bioactive peptides, antioxidant, antithrombotic, Tricine-SDS-PAGE, LC-MS-MS analysis

1. Introduction

Several enzymes are studied for its use in production of biologically active peptides - “bioactive peptides”, as they have great potential (Rao et al. 1998). During the last few decades, it has been found that animal and plant proteins, other than their important nutritional implications, can be used as a source to produce bioactive peptides. The biopeptides liberated, may exhibit various bioactivities such as angiotensin I converting enzyme (ACE) inhibitory activity (Cha et al. 2005), antioxidative (Kim et al. 2001), antimicrobial (Kamysu et al. 2003), antihypertensive (Suetsuna 1998) and immunomodulatory effects (Chen et al. 1995). The protein sources that are highly investigated are soy protein (Gibbs et al. 2004), fish (Kristinsson and Rasco 2000), egg (Park et al. 2001), milk (Gobbetti et al. 2000), and spirulina (He et al. 2006; Ma et al. 2007) proteins. The most commonly used proteases are those from *Bacillus* sp, lactic acid bacteria, and marine yeasts (He et al. 2006; Ma et al. 2007).

Another area in food industry relevant to enzymatic hydrolysis of proteins and formation of low molecular weight peptides are found to be sources of essential amino acids and debittering of protein supplements. Such bioactive peptides are also used to replenish essential amino acids, reduce bitter taste and produce flavour precursors. Therefore, the enzymatic production of amino acid mixtures from proteins using a combination of proteases and peptidases has recently been given considerable attention in the food protein processing industry. The purified protease is of particular interest for biomedical and biochemical applications, and protein hydrolysates in food industry because of their simple production media and condition, less risk of contamination, simple kinetics, possible scaling up and good activity even upon immobilization.

This paper describes the study of applications of this protease in protein hydrolysis for bioactive peptide production. The antioxidant, antimicrobial and antithrombotic properties of peptides obtained from purified protease of *Bacillus* sp. were analyzed. The peptides were characterized and amino acids content were analysed for presence of essential amino acids and debittering amino acids.

2. Materials and Methods

Materials

Amicon Ultra-4 3K centrifugal filter device was procured from Millipore, USA. Casein, 2,2-diphenyl-1-picryl hydrazyl (DPPH); 2,2’-azinobis-(3-thylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, heparin, thrombin, fibrinogen and β-Lactoglobulin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Spirulina was purchased from Parry’s nutraceuticals company; orthopthalaldehyde reagent and amino acids stock standard were procured from

Hewlett Packard. Soy protein was locally purchased and all other chemicals used were of analytical grade purchased either from Merck (Mumbai, India).

Microorganism

The bacterium was isolated from poultry soil and was identified as *Bacillus* sp. MTCC 9558 belonging to *B. cereus* cluster was found to be a good producer of protease reported by us earlier (Prasad et al 2014).

Preparation of protein sample

The purified protease from *Bacillus* sp. was used for hydrolysing different proteins resulting in low molecular weight peptides. The protein rich natural sources such as Spirulina (*Arthospira platensis*); Yeast (*Saccharomyces cerevisiae*) granules; casein; soy protein and β-lactoglobulin were selected based on their protein content and were prepared as protein substrates of protease. Protein concentration in the ultra filtrates of all the samples mentioned above was measured by Lowry et al. (1952).

Enzymatic hydrolysis

The suspensions of different protein sources were mixed with purified protease from *Bacillus* sp. with activity of 5.1 U/ml and the mixtures were incubated in water bath at 45 °C for 6 hours. The reaction was terminated by placing in boiling water bath for 20 minutes. The mixtures were centrifuged to remove the precipitate and the resulting protein hydrolysates were ultrafiltered using Amicon 3 kDa cut off centrifugal filter unit to remove proteins greater than 3 kDa. The filtrate thus obtained was used as peptides sample (Ma et al. 2007).

Determination of antioxidant activity

The DPPH radical scavenging activity was determined (Yen and Wu 1999) with some modification. For DPPH radical scavenging assays, each sample was measured in triplicate and 2 mg/ml ascorbic acid was used as standard. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of antioxidants and of ascorbic acid for the standard reference data.

Effect of time on enzymatic hydrolysis of protein hydrolysates

The proteins from different sources were hydrolyzed with alkaline protease from *Bacillus* sp. and incubated in water bath at 45 °C for different time intervals (2, 4 and 6 hours). Upon inactivation, the effect of time required for complete hydrolysis of proteins by the protease and production of peptides was studied. The mixtures were centrifuged to remove the precipitate, ultrafiltered using centrifugal filter unit to remove proteins greater than 3 kDa and the filtrate was used as peptide sample. The antioxidant activities were measured by DPPH assay.

Determination of antibacterial activity well diffusion method

The potential of the peptides to exhibit antibacterial activity against different pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis* and *Salmonella typhimurium* were observed. This has been reported by us (Prasad et al 2017).

Determination of antithrombotic activity

The peptides of protein hydrolysates by the action of alkaline protease were experimented by their potential to inhibit the thrombin catalysed coagulation of fibrinogen (Yang et al. 2007) with minor modifications. A 0.1% fibrinogen solution and peptides from various hydrolysates of protein concentration 12 mg/ml were added to the wells in the micro titre plates and mixed and the micro titre reader was set to wavelength of 450 nm at 37 °C. The thrombin solution, 10 µl (12 IU/ml; Zhang et al. 2008) was added to initiate reaction of thrombin catalyzed coagulation of fibrinogen. Heparin of different concentrations was used as a positive control and determinations were carried out in triplicates and inhibitory effects were calculated using the equation:

$$\text{Scavenging effect; \%} = \frac{(C - CB) - (S - SB)}{(C - CB)} \times 100\%$$

where S, SB, C, and CB represent the absorbance of the sample, the sample blank, the control, and the control blank, respectively.

Characterization of bioactive peptides

Tricine-SDS-PAGE

Tricine-SDS-PAGE (Shagger 2006); the samples are loaded with Laemmli buffer into the tricine gel wells and after electrophoresis the gel was subjected to silver staining.

Liquid chromatography - mass spectroscopy - mass spectroscopy (LC - MS/MS) analysis

Protein hydrolysates treated with alkaline protease were analysed using LC-MS-MS for peptides. The system was equipped with ultra high pressure chromatographs (UPLC) column for peptide separation of 1.7 µm x 75 µM x 100 mm dimensions. The system was run with column temperature of 40 ^{o}C; flow rate of 0.3 µl/min and gradient elution. [Glu1]-fibrinopeptide B (500 fmol/µl) was infused at 500 nl/min as the reference compound (lock spray). Mass spectrometric analysis of eluting peptides was performed on Synapt G2 HDMS (WATERS).

Amino acid analysis of peptides

The hydrolysates produced by protease were analysed for types of free amino acids, concentration and essential amino acids. All the five peptide samples

resulted from the protease action were run and the chromatogram was obtained for the same. From the chromatogram, the area of the peaks was recorded and was calibrated along with the standards and used for calculations.

3. Results

The culture filtrate of *Bacillus* sp. was purified to homogeneity by hydrophobic interaction chromatography (Prasad et al 2017). The five natural protein rich sources selected were subjected to several treatments and the ultra filtrates obtained with molecular weights more than 3 kDa were used as protein samples. Finally, the protein concentration in all the five samples was adjusted to 0.6 mg/ml and hydrolysed with purified protease from *Bacillus* sp. The protein hydrolysates were ultrafiltered and the peptides less than 3 kDa were used as peptide samples to investigate its antioxidant, antibacterial and antithrombotic activities. The peptides were termed bioactive peptides as they exhibited the above mentioned activities. The bioactive peptides were also characterized and profile of its amino acid content was determined.

Determination of antioxidant activity

Antioxidant activities were measured in all the five peptide samples using DPPH radical scavenging activities, measured as Ascorbic acid Equivalent Antioxidant Capacity (AEAC). Radical-scavenging is the measure of decrease in the absorbance (Shimada et al. 1992). The results suggest that the peptides in different hydrolysates may be different in terms of chain length and amino acid sequence, which gives different DPPH radical scavenging capabilities. Therefore, the protease from *Bacillus* sp. has good potential in production of antioxidant peptides from all the selected protein sources.

Effect of time on enzymatic hydrolysis of protein hydrolysates

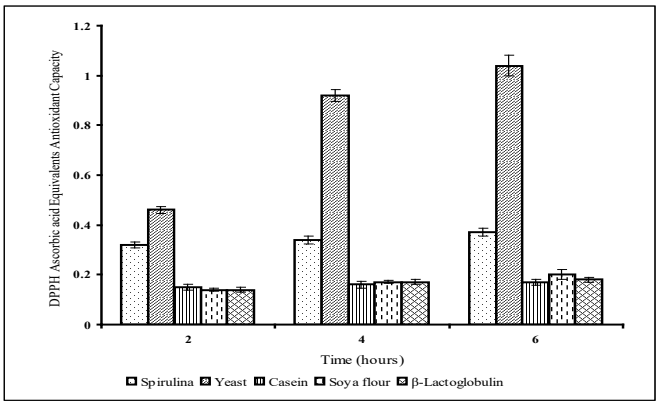

Figure 1. DPPH assay to determine the effect of time on antioxidant activity of peptides from different protein sources

The DPPH radical scavenging activity of protein hydrolysates was studied with respect to time range. The results shown in Figure 1 suggested that antioxidative compounds in the peptide samples tested were capable of radical scavenging to a greater extent when higher time of incubation was given for the protease to react with yeast as protein source with AEAC values in (µg/ml) of 0.46 ± 0.02 at 2 hours; 0.92 ± 0.03 at 4 hours and 1.04 ± 0.04 at 6 hours. Such prominent differences were not observed with other peptides from spirulina, casein, soy and β-lactoglobulin protein hydrolysates produced from the protease activity. Thus, it was confirmed that incubation duration required for bioactive peptide production differed with different protein sources.(Fig 1)

Determination of antibacterial activity – well diffusion method

Casein peptides show antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhimurium. (Fig2)*

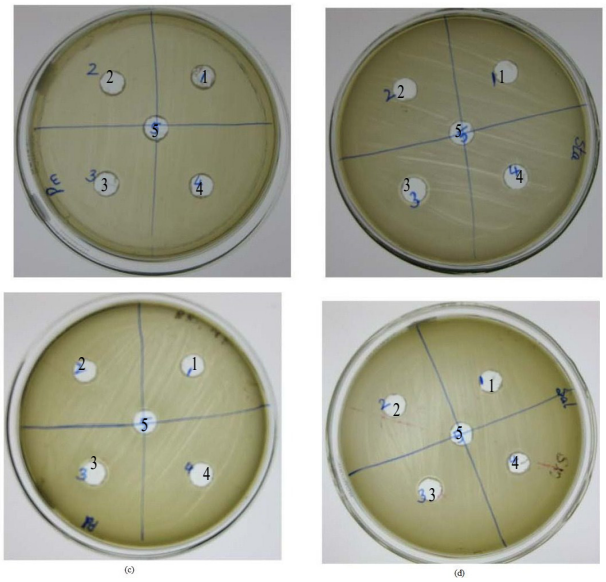

Figure 2. Well diffusion studies for antibacterial activity of bioactive peptides

(a) Bioactive peptides shows no inhibitory activity against Proteus mirabilis;

(b) Staphylococcus aureus ; (c) Pseudomonas aeruginosa and (d) Salmonella typhimurium.

Amongst the five bioactive peptides from different sources indicated are:

1.Spirulina; 2. Yeast cells; 3. Casein; 4. Soy protein and 5. β-lactoglobulin.

Determination of antithrombotic activity

The mechanism of thrombin-catalyzed conversion of fibrinogen is discussed in detail by Scheraga (2004). The results of antithrombotic activity of bioactive peptides from different protein sources are shown in the figure 3. The peptides possessed marked inhibitory activities on the thrombin-catalyzed coagulation of fibrinogen at

concentrations 12 mg/ml. The potent inhibitory effects of about 222.33 ± 2.51% scavenging effect were observed for casein peptides. Heparin, commonly used as an antithrombotic drug, had the potent ability of retarding the formation of the potent ability of retarding the formation of fibrin in a dose dependent manner (Figure 3)

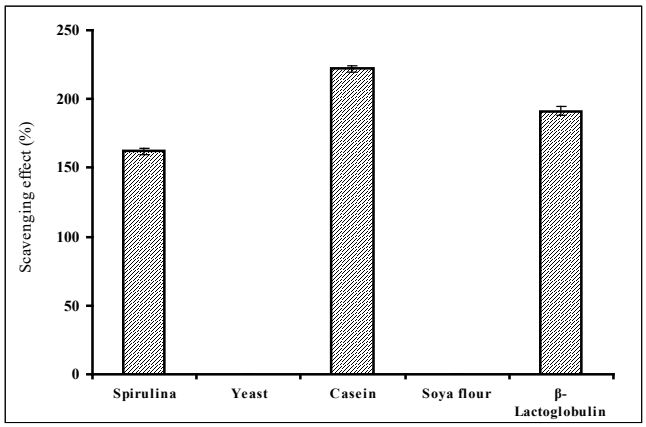

Figure 3. Antithrombotic activity of biopeptides from different protein sources

Tricine-SDS-PAGE for characterizing bioactive peptides

The peptide samples run on Tricine-SDS-PAGE is given in the figure 4. The bands indicate the presence of low molecular weight peptides. The presence of many bands in the first four lanes is due to the protein sources that are crude and the fifth lane is β-Lactoglobulin sample in its pure form. The gel gives an initial confirmation about the presence of low molecular weight peptides that needs further studies (Fig 4).

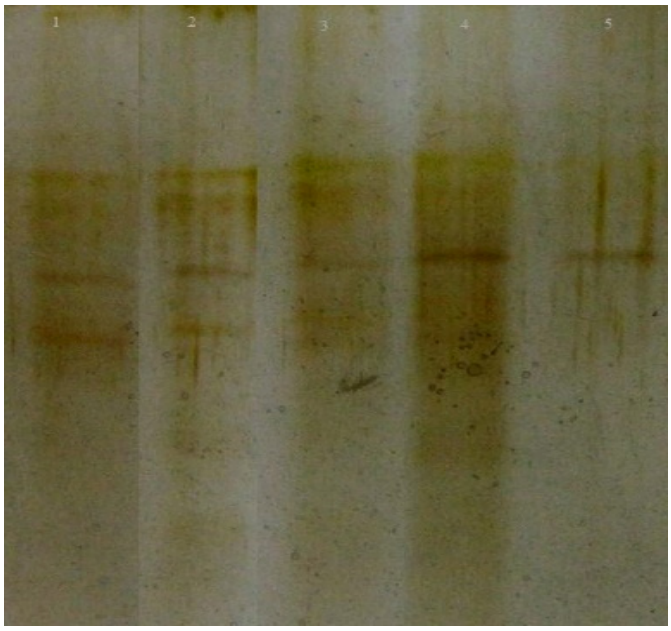

Figure 4. Peptide on Tricine-SDS-PAGE

Table 1: Comparative activities of different protein hydrolysates by the action of protease

Protein hydrolysates by the action of protease	Antioxidant	Antibacterial	Antithrombotic
Spirulina	++	–	+
Yeast	+++	–	–
Casein	+	+++	+++
Soy	+	–	–
β-lactoglobulin	+	–	++

2.+++ indicates maximum activity; **++** next best activity; **+** low activity

LC-MS-MS analysis

The objective of LC-MS-MS bioanalytical is to authenticate the presence of peptides, their molecular weights, which are given below in figure 5. In spirulina protein, the molecular weights of peptides are lesser than 1 kDa and maximum of 1.6 kDa. Only in casein peptides many peptides are in 1 to 2 kDawith peak at 0.7 kDa. These results confirm the molecular weights of peptides. (Fig 5 and Table 2)The amount and types of amino acids released by the *Bacillus* sp. purified protease were monitored and results are shown in Table 2 corresponding to five different bioactive peptides. The free and total amino acids

Table 2. Amino acid profile (amino acid levels in nmoles/ml) bioactive peptides obtained from protein sources upon protease digestion

Amino acids (nmoles/ml)	Spirulina	Yeast	Casein	Soy	β-lactoglobulin
Aspartic acid	143.5	141.7	281.4	272.8	200.2
Glutamic acid	289.6	879	399.8	334.8	364
Serine	91.1	78.5	206.9	157.7	114.1
Histidine	162.9	176.1	180.4	200.7	145
Glycine	212.4	230.4	160.5	94.7	78.7
Threonine	40.3	21.4	94.9	62.9	31.2
Alanine	5295.6	1570	427.1	596	442.8
Arginine	71.2	71.6	94.2	100.8	336.5
Tyrosine	54.7	51.5	417.2	1044	828.6
Valine	120	48.6	2489.2	1911	591.3
Methionine	144.6	107.2	557.4	548.4	601.5
Phenylalanine	95.1	79	240.6	127.5	153.7
Isoleucine	107.6	107.3	304.4	176.2	242.9
Leucine	189.9	288.1	339.8	259.7	388.6
Lysine	104.5	157.5	81.2	100.3	347.9

released by protease treatment in each hydrolysate (6 hours) were analyzed by HPLC. Amino acids, such as Asp, Glu, Ser, His, Gly, Thr, Ala, Arg, Tyr, Val, Met, Phe, Ile, Leu and Lys were the major amino acids quantified for total free amino acids released by all the enzyme treated hydrolysates. The results were analyzed for the

presence of essential amino acids such as His, Ile, Leu, Lys, Met, Phe, Thr, Try and Val. The important essential amino acids content (nmoles/ml) in the peptides is from casein Val 2489; soy protein Tyr 1044 and Val 1911; β-Lactoglobulin Tyr 828, Val 591 and Met 601. The results indicated that these protein peptides have potential applications as food supplements.

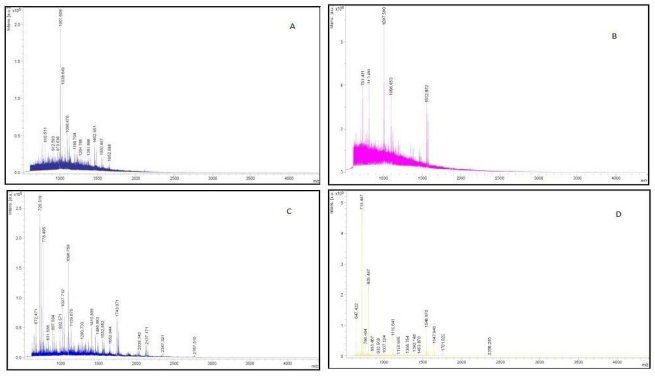

Figure 5. Total ion chromatogram of A) Spirulina B) Yeast C) Casein D) β-Lactoglobulin hydrolysed by protease 4.

Discussion

Antioxidants have been traditionally used in the food processing industries to prevent lipid oxidation (Decker et al. 2005). Young et al. (2010) investigated a number of enzymes and combination of bacterial proteases in order to produce phosphopeptides having antioxidative properties that suggested biological activities of protein hydrolysates that are related to synergistic contributions of amino acid composition, sequence and molecular weight of peptides.

A number of bioactive peptides have been identified in milk proteins, such as casein and whey proteins, where they are present in an encrypted form, stored as propeptides or mature C-terminal peptides that are only released upon proteolysis (Kamysu et al. 2003). Bioactive peptides had lesser potency of antithrombotic activity comparable to heparin, but, they had the advantage of having no known side effects. The pioneer work on sequencing of synthetic short peptides was done by Laudano and Doolittle (1978), that the sequence of Gly-L-Pro-L-Arg, which corresponded to the amino(N-)-terminal segment of the fibrin a chain after the release of the fibrinopeptide A (FpA) by thrombin, could bind to fibrinogen and prevent the polymerization of fibrin monomers.

LC-MS-MS analysis is also used to determine the amino acid sequence and type of proteins. Deng et al. (2010) has used tandem mass spectrometry to obtain partial amino acid sequence of AprB protease from *Bacillus* sp. B001. This technique is also used for peptide mass fingerprinting of purified peptides.

The bitter taste removal is an important criterion in food industry. Several attempts have been tried to reduce this bitterness by hydrolyzing the bitter peptides with exoproteases (Minagawa et al. 1989). The hydrobhoic amino acids such as Val, Leu, Phe and Try content are responsible for bitter taste of proteins (Ishibashi et al. 1988). As the pro tease hydrolyzes peptides containing hydrophobic amino acids, they are much useful in reducing the bitterness by removing these amino acids. Therefore, Bacillus sp. protease could have the scope to eliminate the bitter taste by selective hydrolysis of bitter peptides and could offer possibilities for industrial applications, including debittering of protein hydrolysates.

4. Conclusions

Thus, the significant emphasis has been given to the development of safe and effective food supplements and serving various bioactivities from abundant natural sources, mainly peptides derived from hydrolyzed food proteins. The factors which affect the bioactivities are the operational conditions employed in the processing of protein isolates, the type of protease, and the degree of hydrolysis. Especially, the proteinase used can affect both the functional properties and other activities of the protein hydrolysates obtained. Moreover, the utilization of proteins or their hydrolysates for food and cosmetic applications not only provides additional advantages butalso confers nutritional and functional properties.

Conflict of interest None

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