Current Trends in Biotechnology and Pharmacy
Vol. 15 (6) 114 -117, 2021, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2021.6.20

Substrate Specificity of Paraben Towards Liver Esterase: An *In-Silico* and Titrimetric Analysis

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Abstract

Parabens, esters of para-hydroxybenzoic acid, have so far been "safely" used in our daily life as a preservative in most of the commercial products including cosmetics. It is the relatively inert, non-reactive, odorless nature and easy miscibility with the majority of the products that make it a suitable choice as preservative. However, recent studies have explored the dark side of the uses of this compound, as a potential environmental hazard due to its impact on humans as endocrine disruptor and carcinogen. In current study *in-silico* interactions between one such most extensively used Paraben, methyl Paraben (MP) and Porcine liver esterase were explored. Based on docking studies and binding energies of substrate (ethyl butyrate) and MP, it can be proposed that Porcine liver esterase can be a potential enzyme for MP hydrolysis. Also, the titrimetric method for esterase assay was also performed. The kinetic parameters, analysed using Line WeaverBurk's plot showed K_m and V_{max} to be 0.08 mM and 400 U/ml, respectively. The studies proposed that MP can be a potential substrate for hydrolysis by Porcine liver esterase, at pH 8.0 and 25 ºC. The study will open the avenue of using enzymes for designing analytical tools for detection of Paraben.

Keywords Paraben, Preservative, Docking, Esterase, Binding energy.

Introduction

Globalization and urbanization has led to the development of modified user friendly products, often with better shelf life. Some of the daily life products including cosmetics, packed and processed food and drugs are some such products that have shown improved shelf life due to supplementation with preservatives, a diverse array of which commonly exist and are used for packaged food, pharmaceutical, cosmetic products and daily used products. They are the esters derivatives of 4-hydroxybenzoic acid as side chains. The presence of Paraben in nature has been well documented (e.g. blueberries, cloudberry, yellow passion fruit), but at relatively low concentrations [6]. It was reported that the concentration of methylparaben in *Andrographis paniculata* is around 0.0008% of its weight [8]. Alternatively, owing to their vast applications, Parabens are also produced synthetically in industries, as they have a number of good properties to use as a preservative [1]. Some of the common properties that obtrude paraben as a well-accepted preservative in food, cosmetic, pharmaceutical and other similar products are their broad spectrum of activities against bacteria, yeasts and other microbes, chemical stability (for a wide temperature interval and pHs ranging from 4.5 to 7.5), low frequency of sensitization, low degree of systemic toxicity, adequate water solubility that enables formation of effective concentration and no perceptible odor. Moreover, Parabens were considered relatively safe use with low production cost, maintains consistency and also, does not change coloration of products.

Analysis of Paraben stability in acidic and alkaline conditions showed that the compounds are hydrolyzed into p-hydroxybenzoic acid (pHBA) and corresponding alcohol in alkaline conditions and that parabens are highly stable in acidic aqueous solutions [10].

However, the negative impacts of Parabens on human health had been explored lately. Studies have shown that Parabens, used in cosmetics, can penetrate through the skin directly into the human body [6]. The average daily exposure of total paraben is estimated to be 1mg from food, 50 mg from personal care products and cosmetics products accounting for 76 mg and 25 mg on consuming pharmaceuticals on an individual level [10].

Parabens used in cosmetics creams/lotions are broken down by keratinocyte carboxylesterases enzyme present in the skin and other esterases enzyme within the gastrointestinal tract and liver; then the conjugated metabolites are excreted in bile and urine [6]. This can be influenced by the presence of penetration enhancers found in cosmetic preparations [7]. Though, the presence of carboxylesterases in skin and subcutaneous fatty tissues results in varying hydrolysis to *p*-hydroxybenzoic acid [9] and this influences their absorption [2]. Through various ways, the group of paraben gets bioaccumulated and biomagnified through food chains and web due to the relatively high lipophilicity [14].

The quest to detect parabens in different products and environmental samples, relies on development of specific analytical tools. Such analytical tools and prototypes can be created with availability of efficient detection mechanism. Studies from Khalid (2019), had reported the development of electrochemical sensor for detection of methyl parabens [5]. Yücebaş et al., (2020) reported antibodies based biomimetric electrochemical biosensors [15]. Current study focuses on exploring the catalytic activity of pig liver esterase towards Methyl Paraben (MP). Studies by Hasenpusch et al., (2012) reported the amino acid triads of the active site of pig liver esterase being guarded by amino acid Serine 204 and Histidine 449 [4]. In current study, the active site interaction studies were performed using Autodock Vina software [11]. The results were analysed and the binding energy of MP was compared with ethyl butyrate (substrate), catechin (positive control), benzene (negative control). The enzymatic catalysis is often regulated by the transition state of enzyme substrate complex. The dynamic changes in the structure of enzyme cannot be validated by Autodock vina studies. Further,

validation of Paraben catalysis by pig liver esterase was confirmed using titrimetric assay methods.

Materials and Methods

Chemicals and Reagents

The porcine (pig) liver esterase was purchased from Sigma Aldrich, India. The enzyme activity, reported by manufacturer was "One unit will hydrolyze 1.0 µmole of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25 °C". Methyl Paraben was purchased from CDH Fine Chemical (P) Ltd., India. Ethyl butyrate used as substrate for esterase assay was purchased from Himedia Laboratories Pvt. Ltd., India. The sodium hydroxide (NaOH) used in the titrimetric assay was purchased from Himedia Laboratories Pvt. Ltd., India.

In-silico docking studies

The in-silico docking studies of MP, Catechin, ethyl butyrate and benzene were performed using Autodock Viva open-source software [11]. The docking of the Porcine Liver Esterase (PDB ID: 5FV4) with grid box around serine 204 in A chain was prepared and docked with the MP (Pubchem CID: 7456), ethyl butyrate (substrate; Pubchem CID: 7762), catechin (positive control; Pubchem CID: 9064) and benzene (negative control; Pubchem CID: 241). The ligand molecules were downloaded from Pubchem data repository (https://pubchem.ncbi.nlm.nih.gov/) as 3D SDF conformer and saved as pdb file using Pymol freeware (downloaded from https://pymol.org/). The Porcine liver esterase molecules were prepared by deleting water, adding polar hydrogen atoms and adding kollman charges, using Autodock tool and grid box was arranged around the selected amino acid Serine 204, reported to be present in the active site of enzyme for catalysis [4]. The binding energies of the docking studies were compared. The results were analyzed using PyMOL.

Titrimetric Assay of Porcine (pig) liver esterase

The catalytic activity of Porcine liver esterase on methyl Paraben was estimated using titrimetric assay method, reported by the manufacturer of enzyme (Sigma Aldrich) and by Harrer et al., (1941), with modification [3]. Ethyl butyrate was used as substrate. The titrimetric method involved use of pH meter (Thermo Scientific, Eutech Instruments, India) with probe immersed in beaker with reaction mixture, to observe the change in pH during enzyme catalysis. The reaction mixture was prepared by mixing substrate, buffer and enzyme in the beaker, placed on magnetic stirrer. The reaction mixture had 9ml of borate buffer (10mM buffer in 1N NaOH), 1ml of substrate and 0.25ml of 0.1% Bromothymol blue dye, used as indicator. The pH of the reaction mix was adjusted to 8.0 using 0.01N NaOH, present in the burette. Then, 1ml of enzyme (50 units/ml in cold borate buffer) was added into the mixture. The esterification of substrate produced acids that decreased the pH of the mixture. The pH was adjusted by adding NaOH, present in the burette. The studies were performed for 10 minutes. The titrimetric assay was performed with absolute (100%) ethyl butyrate and varying concentration of MP (0, 0.02, 0.04, 0.06, 0.08, 0.1mM).

Enzyme Kinetics

The catalytic activity and Michaelis menten's constant of the enzyme towards MP was estimated using Line WeaverBurk's plot.

Results and Discussion

The growing concern over Paraben mediated health hazards had attracted scientific intervention in the past years. Researchers are exploring the possible biological methods for degradation of Paraben [12]. They reported the existence of gene *prbA*, coding for an enzyme hydrolyzing parabens in *Enterobacter cloacae* and *Enterobacter gergoviae*.

The docking of Porcine liver esterase with the different substrates (Methylparaben, Catechin, Benzene and Ethyl Butyrate) are analysed using PyMOL (Fig.1). The binding energies of the substrates and enzyme were analysed, the positive control Catechin, showed the highest average binding affinity of -7.0 kcal/mol while the negative control, benzene, showed the average binding energy of -4.0 kcal/mol. The substrate, ethyl butyrate, has binding affinity of -4.7 kcal/mol, which is similar to the binding energy of methylparaben (-4.5 kcal/mol). Based on this analysis, it is proposed that there is a possible hydrolytic interaction between the Porcine liver esterase and methyl paraben.

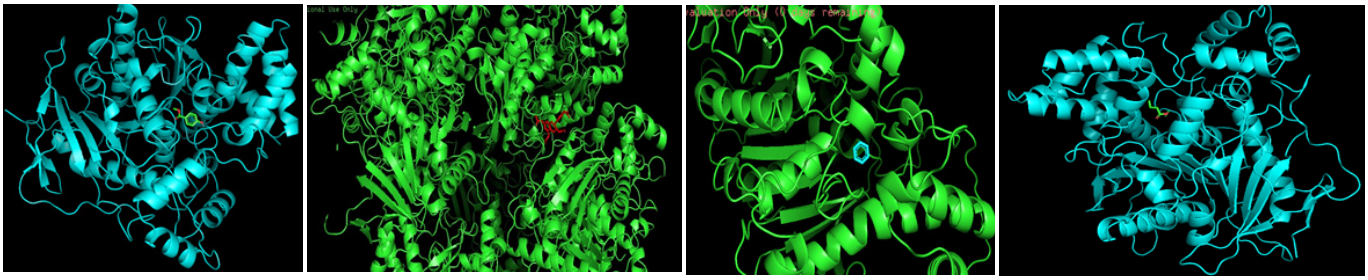

Fig.1: PyMOL visualization of the docking of Pig Liver Esterase with a) Methylparaben, b) Catechin, c) Benzene and d) Ethyl butyrate

Further, the catalytic activity of Porcine liver esterase was estimated using titrimetric assay method. The enzyme activity depends on volume of NaOH used to neutralize the free acid liberated as a result of enzymatic hydrolysis of MP (Table 1). The enzyme followed Michaelis Menten's kinetics analysed in the plot of substrate versus activity (Fig.2). The Equation 1 was used to estimate kinetic parameters of the enzymes.

Table 1: Observations of titrimetric assay of Porcine liver esterase mediated hydrolysis of methyl paraben

Substrate used	Conc. of Substrate used	pH value after addition of substrate	Vol. of NaOH used (ml)	Enzyme Activity (U/ml)
Ethyl Butyrate	Absolute	8.02	24	2400
Methylparaben	0.02mM in 100% water	8.07	0.075	75
Methylparaben	0.04mM in 100% water	8.0	0.1	100
Methylparaben	0.06mM in 100% water	8.02	0.2	200
Methylparaben	0.08mM in 100% water	8.0	0.2	200
Methylparaben	0.1mM in 100% water	8.01	0.2	200

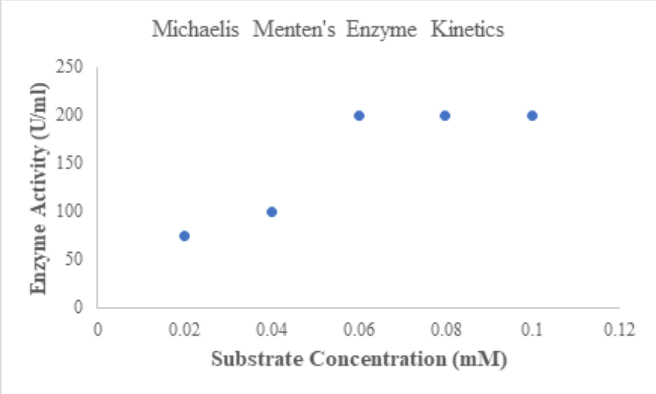

Fig.2: Graphical representation of Michaelis Menten's enzyme kinetics of Procine liver esterase towards MP

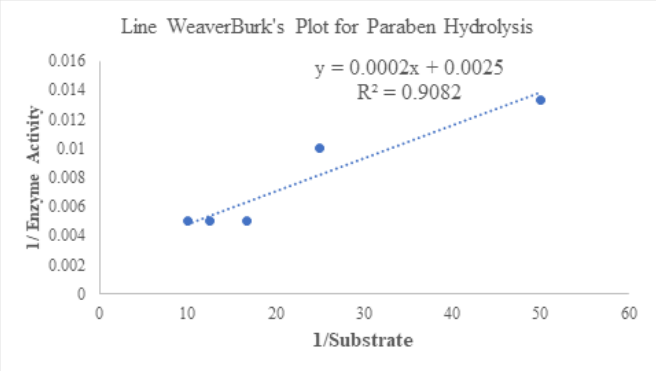

Fig.3: The graphical representation of Line WeaverBurk's Plot for Paraben hydrolysis by Porcine liver esterase

The kinetic parameters analyzed using Line WeaverBurk's plot (Fig.3) showed the Michaelis Menten's constant (K_m) to be 0.08 mM and maximum enzyme activity (V_{max}) of 400 U/ml. The plot have a increasing trendline with R^2 of 0.9 showing a good rate of hydrolysis of paraben with the enzyme. The kinetic parameters *viz.* V_{max} and K_m, in this study at pH 8 and 25 ºC, were observed to be higher than those reported previously [13]. The possible variations in the kinetic parameters, due to different optimum pH, temperature and sources of enzymes, cannot be ruled out. The studies may be further extended to analyze catalytic activity of the enzyme at various pH and temperature regime. The study may be further extended to explore the use of the proposed Porcine liver esterase as analyte for detection of Methyl Paraben.

Conclusion

The growing awareness of consumers towards the possible harmful effects of commonly used preservative compounds Parabens, had shifted the impetus toward exploration of alternatives preservatives, and ways to minimize their use in various products. However, in the absence of suitable alternative, Parabens are still on the list of commonly used preservatives in most of the food, pharmaceutical and cosmetic products. Also, their existence in environment may impart a major threat to the mankind, due to possible health impact. The study aimed at exploring the role of esterase on hydrolysis of MP, one of the most commonly used Paraben. Estimation of kinetic parameters had shown the hydrolysis of MP with the enzyme. Further analysis may help researchers, explore Porcine liver esterase as a potential enzyme for the hydrolysis of MP, a strategy that may be pivotal in MP degradation and analysis.

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