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Effective detoxification of patulin from aqueous solutions by immobilized porcine pancreatic lipase

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ABSTRACT

Porcine pancreatic lipase (PPL) was immobilized by physical adsorption onto $CaCO_3$. The detoxification of the immobilized PPL for patulin from aqueous solutions was investigated. Batch experiments were carried out to obtain its optimal conditions for the immobilization process and to evaluate the influence of the immobilized PPL amount, pH, temperature, initial patulin concentration and time on the detoxification. Enzyme properties were assayed by the immobilization yield and enzyme activity, and partial structures of the immobilized PPL were characterized by Fourier Transform Infrared spectroscopy (FTIR). The immobilization yield was more than 99% during 3 h at pH 5.0, 30 °C when 0.6 mg/mL of PPL was adsorbed onto 0.5 g of $CaCO_3$. The immobilized PPL was effective in detoxification of patulin from aqueous solutions at pH 6.0, 40 °C during 42 h. Furthermore, the immobilized PPL after detoxification still kept high yield and enzyme activity, and was reusable for usage of detoxification. Detoxification properties of the immobilized PPL could represent a novel strategy for a possible application in decontaminating patulin aqueous solutions.

1. Introduction

Mycotoxin is a kind of fungal metabolites, which can contaminate agricultural products, resulting in economic loss and worldwide health threats. Patulin (4-hydroxy-4H-furo[3, 2c] pyran-2 [6H]-one) is a water-soluble secondary metabolite produced by a variety of fungi genera, in particular, *Aspergillus*, *Penicillium* and *Byssochlamys* (Steiman, Seigle-Murandi, Sage, & Krivobok, 1989). Chemically speaking, as a highly reactive polyketide heterocyclic lactone, patulin is heat-stable (Stott & Bullerman, 1975), which showed acute and chronic toxicities for both animals and humans at a very low dose, leading to various acute, chronic and cellular level of health hazards (Glaser & Stopper, 2012; Mahfoud, Maresca, Garmy, & Fantini, 2002). Consequently, a growing number of countries and organizations have realized the importance of establishing related regulations and have specified tolerable levels of patulin in a plenty of susceptible products. For example, according to a NOEL (no observable effect levels) of 43 μg/kg bw/day and a safety factor of 100, a PMTDI (provisional maximum tolerable daily intake) of 0.4 μg/kg body weight/day was set by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) (JECFA, 1995). The Codex Alimentarius Commission (2003) adopted the maximum concentration for patulin at 50 μg/kg in apple juices. Additionally, 50 μg/kg of patulin was set as the maximum permitted level for apple and hawthorn products in 2011 by the new National Food Safety Standard-Standards for Mycotoxins in Food in China. (Guo, Zhou, Yuan, & Yue, 2013; Ministry of Health of the People's Republic of China, 2011).

Patulin contaminations mostly occur in the rotten parts of foodstuffs. Apples and their products are of the greatest concern, while other fruits such as pears and grapes also suffer occasionally. Furthermore, some vegetables and cereals are susceptible to patulin contamination as well, though to a less extent (Sant'Ana, Rosenthal & de Massaguer, 2008; Zouaoui, Sbaii, Bacha, & Abid-Essefi, 2015). Generally, the existence level of patulin can be viewed as a standard to evaluate food quality and the efficiency of pre-/post-harvest practices (Janotová, Čížková, Pivoňka, & Voldřich, 2011; Liu et al., 2015; Vaclavikova et al., 2015). Because of the detrimental effects of patulin, several strategies have been developed to decontaminate food and feeds. However, the limitations of

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physical and chemical methods, such as quality loss, high cost, chemical residual during the process and environmental pollution make it extremely difficult for practical applications (Fernández-Cruz, Mansilla, & Tadeo, 2010; Hao, Zhou, Koutchma, Wu, & Warriner, 2016; Moake, Padilla-Zakour, & Worobo, 2005). Although microbial detoxification had extensively studies (Castoria et al., 2011; Topcu, Bulat, Wishah, & Boyacı, 2010; Zhu et al., 2015), the process of microbial detoxification contained its metabolism and the reduction of mycotoxin. The microbial metabolite is more complex and difficult to detect and separate from the food, which make food quality low. Therefore, none really fulfills the necessary efficacy and safety in the food industrial application (Liu, Sui, Wisniewski, Droby, & Liu, 2013). Enzyme is biocatalyst, which exhibits excellent substrate specificity and could be operated under mild reaction conditions (Yang et al., 2013). Moreover, enzyme treatment had been reported in the detoxification of mycotoxin, such as Zearalenone (ZEN) (Takahashi-Ando et al., 2004), Aflatoxin B_1 (AFB_1) (Taylor et al., 2010). But there is no information concerning enzyme treatment detoxification of patulin. Therefore, seeking a novel enzyme for patulin detoxification is an urgent need.

Lipases are a large family of hydrolases ubiquitously found in living organisms, and have been widely used in biotechnology, food and pharmaceutical industries (de Miranda, Miranda, & de Souza, 2015; Yang et al., 2013). Porcine pancreatic lipase (PPL) is one of the most extensive lipases used for transesterification and biotransformation reactions due to its accessibility, high stability and broad specificity for biotransformation of non-natural substrates, containing six disulfide bridges and two free thiol groups, the catalytic triad of which is composed of serine (Ser), histidine (His) and aspartate (Asp) (Mendes, Oliveira, & de Castro, 2012). Taking economic and technological factors into consideration, the advantages of immobilized PPL overweigh that of the free one, including strong stabilities, ease of separating and recycling, excellent specificity and catalysis (Zhu et al., 2014). Besides, supporting materials, such as $CaCO_3$, have the satisfying biocompatibility, large interfacial area (Bai & Zhou, 2004; Shan, Zhu, Xue, & Cosnier, 2007).

The objective of this study was to prepare the immobilized PPL and reduce the levels of patulin from aqueous solutions. The optimal immobilization conditions and the detoxification parameters were discussed. The reusability and partial characterization of the immobilized PPL were also carried out in attempt to interpret the mechanism.

2. Materials and methods

2.1. Materials

The porcine pancreatic lipase (type II, EC 3.1.1.3, with a specific activity of 100–400 olive oil units per milligram of protein) was supplied by Sigma-Aldrich (St. Louis, USA). A commercial standard of patulin was purchased from Sigma-Aldrich Co. Ltd (Shanghai, China). Olive oil was produced by Shanghai Lanji Technology Development Co. Ltd. Calcium carbonate ($CaCO_3$) was of analytical grade and obtained from Tianjin Basf Chemical Co. Ltd (Tianjin, China). Gelatin, SBA-15, celite, activated carbon, coomassie brilliant blue G250 (CBBG), ethanol, acetate, sodium hydroxide and ethyl acetate were of analytical grade and used as received without further purification. Polyvinyl alcohol was of analytical grade and acetonitrile was of chromatographic grade. All solvents employed were prepared with deionized distilled water.

2.2. Immobilization of PPL on different support materials

Five different support materials were used for immobilization: $CaCO_3$ (Ghamgui, Miled, Karra-chaâbouni, & Gargouri, 2007), gelatin (Wang et al., 2011), SBA-15 (Yang et al., 2013), celite (Khare & Nakajima, 2000) and activated carbon (Brito et al., 2016).

0.5 g of PPL was dissolved in 500 mL of phosphate buffer solutions (pH 6.81, 33 mM) by stirring in the vessel at 4 °C, and obtained supernatant, known as the enzymatic solution. Then 10 mL of enzymatic solution (1 mg/mL) were mixed with 0.5 g of different support materials in a conical flask, and the mixture kept under continuous agitation in an orbital shaker at 180 rpm for 12 h at 40 °C. After immobilization, supernatants and solids were separated by centrifugation (5000 rpm, 10 min). Supernatants were assayed to give the amount of enzyme protein by Bradford's method (Bradford, 1976) and determined for enzyme activity residue. Subsequently, 10 mL chilled acetone was added to solids, and the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 10 mL aliquot of chilled acetone and lyophilized under vacuum.

2.3. Enzymatic properties assay

2.3.1. Protein content and yield of immobilization

Protein content was determined according to the methodology described by Bradford (1976), using bovine serum albumin as the standard.

The amount of support loading (mg/g) was calculated by Eq. (1):

$$p = \frac{\left(C_0 - C_f\right) \times V}{M} \tag{1}$$

where, p is the amount of support loading (mg/g, mg enzyme per gram support); C_0 is the initial amount of enzyme protein offered (mg/L), and C_f is the amount of enzyme protein in the supernatant of the immobilization (mg/L); V is the volume of the reaction medium (L); M is the weight of the supports (g).

The yield of immobilization (η) was calculated by Eq. (2):

$$\eta(\%) = \frac{C_0 - C_f}{C_0} \times 100 \tag{2}$$

where, η is the yield (%); C_0 is the initial amount of enzyme protein offered (mg/L), and C_f is the amount of enzyme protein in the supernatant of the immobilization (mg/L).

2.3.2. Hydrolytic activity assay

Hydrolytic activities of immobilized PPL were assayed by the olive-oil emulsion method (Silva et al., 2014), with slight modifications.

The substrate was prepared by mixing 5 mL of olive oil with 15 mL of polyvinyl alcohol solution (2%, m/v). The reaction mixture contained 5 mL of the emulsion, 3 mL of 0.025 mol/L buffer sodium phosphate at a pH of 7.5 and 0.10 g of the immobilized lipase, were under continuous agitation in an orbital shaker (200 rpm) for 15 min at 40 °C. The reaction was stopped by adding 15 mL of ethanol (95%, v/v). The liberated fatty acids were titrated with 0.05 mol/L sodium hydroxide solution in the presence of phenolphthalein as an indicator. The reaction blanks were carried out by repeating the above assay procedures in the absence of immobilized lipase after the ethanol (95%, v/v).

In this study, PPL activity unit (Us) was defined as the amount of free fatty acid, produced by the hydrolysis reaction with 1 g of immobilized lipases under the conditions described above, and it was regarded as 100 units, consuming 1 mL of 0.05 mol/L NaOH for free fatty acid.

Consequently, the hydrolytic activities of immobilize PPL was