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Preparation of a Crosslinked Bioimprinted Lipase for Enrichment of Polyunsaturated Fatty Acids from Fish Processing Waste

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Abstract *Geotrichum* sp. lipase modified with a combined method composed of crosslinking and bioimprinting was employed to selectively hydrolyze waste fish oil for enrichment of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in glycerides. Crosslinked polymerization by monomer (polyethylene glycol 400 dimethyl acrylate), crosslinker (trimethylolpropane trimethylacrylate), and photoinitiator (benzoin methyl ether) coupled to bioimprinting using palmitic acid as imprint molecule, resulted in much more effective enzyme preparation used in aqueous hydrolysis reaction. Since the crosslinked polymerization modification maintained bioimprinted property and gave good dispersion of enzyme in reaction mixture, the crosslinked bioimprinted enzyme exhibited higher hydrolysis temperature, enhanced specific activity, shorter hydrolysis time, and better operational stability compared to free lipase. Crude fish oil was treated at 45 °C with this crosslinked bioimprinted lipase for 8 h, and 46% hydrolysis degree resulted in the production of glycerides containing 41% of EPA and DHA (EPA+DHA), achieving 85.7% recovery of initial EPA and DHA. The results suggested that bioimprinted enzymes did not lose their induced property in aqueous environment when prepared according to the described crosslinking–bioimprinting method. It could also be seen that the crosslinked bioimprinted lipase was effective in producing glycerides that contained a higher concentration of polyunsaturated fatty acid with better yield.

Keywords Lipase · Crosslinking · Bioimprinting · Hydrolysis · Polyunsaturated fatty acid

Introduction

Design of enzyme preparations with high activity for use in aqueous or nonaqueous reaction has attracted increasing attentions due to its prominent performance over native

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enzyme and chemical catalysts [1]. Immobilization, lipid coating, and bioimprinting are often employed to modify lipase [2–10]. Among these methods, bioimprinting is a convenient and feasible method for improving lipase performance in organic solvent and solvent-free systems [5, 6, 11]. The general bioimprinting principle is as follows. When imprint molecules (substrates or substrate analogs) are added to lipase solution, an interface is introduced to activate opening of the lipase's lid structure and remaining of cavity fitting of the catalytic machinery, which facilitates matching of substrate functional groups after the imprint molecules are removed. The induced conformation is then trapped by rapid freeze-drying and is retained in anhydrous organic solvents. Therefore, bioimprinted lipase exhibits higher catalytic activity in nonaqueous reactions compared to corresponding free enzyme form [5]. Unfortunately, this method is limited by the fact that the induced properties are available in nearly anhydrous environment only since aqueous environment causes a renaturation of the protein and loss of the imprinting effect [5]. In an effort to maintain the imprinted properties of proteases in an aqueous environment, bioimprinted enzymes were covalently stabilized by first vinylated and then crosslinked with ethylene glycol dimethylacrylate to form a rigid conformation for use in aqueous as well as inorganic environment [12]. In a previous study, we described the use of a combined modification method including bioimprinting, pH tuning, lipid coating, salt activation, and immobilization for improving lipase's activity in nearly anhydrous organic solvents [13]. The main interest of this work was to stabilize imprinted lipase property by an alternative technique based on crosslinked polymerization for use in aqueous environment.

Fish oil contains polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are important bioactive compounds used in biomedical field [14, 15]. Enrichment of PUFAs from fish oil based on lipase-catalyzed reaction including hydrolysis, esterification, and trans-esterification had been extensively performed [16–22]. However, enrichment work based on waste feedstock is poorly understood. Large quantities of by-products such as head and internal organs are produced during the marine fish processing, and usually regarded as the waste. The waste by-products are the potential resource since they contain certain amounts of PUFAs [23]. In the present work, *Geotrichum* sp. lipase modified by combining imprinting with crosslinking was employed to selectively hydrolyze fish oil from waste for enrichment of EPA and DHA in glycerides.

Materials and Methods

Lipase from recombinant *Pichia pastoris* GS115 containing a *Geotrichum* sp. lipase gene was produced and purified as described by Yan et al. [24]. Neutral protease 1398 (food grade, 100,000 U/g) was purchased from Wuxi Enzyme Preparation Company (China). Polyethylene glycol (400) dimethacrylate (PEGDA), trimethylolpropane trimethylacrylate (TMPTA), and benzoin methyl ether were all analytical grade purchased from a chemical corporation. All internal standards were purchased from Sigma.

Preparation of Crude Fish Oil

Fresh fish (tuna, salmon, etc.) processing wastes such as head and internal organs were collected from Guangdong fishing industry and used as initial feedstock. After cooking for 30 min at 85 °C, the materials were separated into minced fillet and bone with bone

trimmer and mincer. Preparation of crude fish oil from fresh fish processing waste was performed as follows: following adjusting pH to 7.5, a sealed mixture of minced fillet/water (1:1, *w*/*w*) and 1,000 U/(g minced fillet) neutral protease filled with nitrogen was incubated at 45 °C with stirring at 300 rpm. After enzymatic hydrolysis for 6 h, the reaction solution including oil phase and aqueous phase was obtained by removing solid residue. The upper oil phase was obtained by centrifugation for 5 min at 4,500 rpm, called crude fish oil. The main fatty acid composition of crude oil was determined in Table 1.

Preparation of Crosslinked Bioimprinted Lipase

The mixture containing monomer polyethylene glycol 400 dimethyl acrylate (40 g) and 2.5 g of imprint molecules (palmitic acid) was incubated at 37 °C until dissolved. Thirty milliliters of enzyme solution (10 mg/ml) was added dropwisely into the mixture and gently stirred until the mixture became homogeneous microemulsion system. Then trimethylolpropane trimethylacrylate (20 g) as crosslinker and appropriate amount of benzoin methyl ether as photoinitiator were added into the above mixture for polymerization induced by exposion under UV for 45 s. When polymerization process finished, the polymers were washed with water and acetone to remove unreacted materials and imprint molecules, and the polymers were vacuum dried to remove the solvent. After that, the polymers containing lipase were ground into powders followed by screening with 20–40 mesh sieve for use. The powder preparation with particle diameter of 420–840 μm, specific surface area of 760–810 $m^2\ g^{-1}$ was called cosslinked bioimprinted lipase (CBL). Cosslinked lipase (CL) was prepared by addition of PEGDA and TMPTA, but without addition of the imprint molecule. Bioimprinted lipase (BL) was prepared by addition of the imprint molecule, but without addition of PEGDA and TMPTA. Free lipase (FL) without addition of any additives was used as a control. The protein content of enzyme preparations was determined using the Bradford method with BSA as the standard [25].

Lipase Activity Assay

Activity determination was carried out titrimetrically as described previously using 50 mM NaOH as titrant [26]. The reaction mixture contained 5 ml of 50 mM Tris–HCl buffer

Table 1 Content of main fatty acid in the glycerides before and after hydrolysis.

Fatty acid (%)	Original	After hydrolysis[a]	
		Free lipase (14 h)	CBL (8 h)
Palmitic acid ($C_{16:0}$)	32.7	28.4	15.8
Palmitoleic acid ($C_{16:1}$)	13.4	10.3	7.4
Stearic acid ($C_{18:0}$)	8.6	8.9	8.8
Oleic acid ($C_{18:1}$)	16.3	11.2	9.2
EPA ($C_{20:5}$)	4.2	5.9	7.1
DHA ($C_{22:6}$)	17.8	24.3	33.9
Hydrolysis degree	–	25.7%	46%

[a] The hydrolysis reactions were performed in optimal conditions by CBL and FL, respectively

(pH 8.0), and 4 ml of olive oil as substrate. Unless stated otherwise, incubations were carried out at 40 °C and pH 8.0 for 10 min. The enzymatic reaction was initiated by addition of various enzyme preparations to the reaction mixture and stopped by the addition of 15 ml of ethanol. The control was carried out similarly, except that the enzyme solution was added after the addition of ethanol. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 μmol of free fatty acid from olive oil per minute under test conditions.

Selective Hydrolysis of Crude Fish Oil with Various Enzyme Preparations

In preliminary experiments, a mixture containing 3 g of crude fish oil, 3 ml of deionized water, and 100 U/(g reaction mixture) of various lipase preparations was incubated at 30 °C with stirring at 300 rpm. At designated time, 15 ml of ethanol was added, and the acid value was measured by titration with 0.4 mol/l KOH. The extent of hydrolysis was measured as follows:

Hydrolysis degree $= \frac{AV_t - AV_0}{SV - AV_0} \times 100\%$, where SV and AV_0 are the saponification value and acid value of the original crude fish oil, respectively, and AV_t is the acid value of the reaction mixture at designated times [27]. Fractionation of glycerides in reaction mixture was extracted with 100 ml *n*-hexane after addition 50 ml of 0.5 mol/l ethanolic KOH to the hydrolysis reaction mixture.

Fatty acids in glycerides were methylated with sodium methylate. These methyl esters were analyzed by gas chromatography (GC-9790) equipped with flame-ionization detector and a capillary column (INNOWAX, Agilent, 30 m×0.25 mm×0.25 μm). The oven temperature was increased from 150 to 210 °C at the ramp of 3 °C/min, and the temperatures of injector and detector were both set at 250 °C. The helium was used as the carrier gas. Fatty acids were identified and quantified by comparison with standards.

Results and Discussion

Comparison Effect of Different Enzyme Preparations on Selective Hydrolysis

As shown in Fig. 1, the four enzyme preparations (CBL, CL, BL, and FL) were applied to selectively hydrolyze the crude oil for enrichment of DHA and EPA. Distinctly, the CBL achieved marked hydrolysis degree (41.9%) after 12 h reaction, while CL, BL, and FL reached only 30.8%, 24.5%, and 23% after 15 h reaction, respectively. The significant enhancement in hydrolysis degree using CBL as a biocatalyst was attributed to dual treatment with bioimprinting and crosslinking. CL modified with only crosslinking promoted the hydrolysis to a certain degree compared to the case of FL. However, BL with only single treatment by bioimprinting almost had no positive effect on hydrolysis degree. Although bioimprinting is a convenient and feasible method to improve enzyme preparations with desirable catalytic properties, the bioimprinted properties are available in nearly anhydrous environment only since aqueous environment causes a renaturation of the protein and loss of the imprinting effect [5]. In the case of BL for hydrolysis reaction in aqueous environment, water present in reaction mixture diminished the activation effect of bioimprinting by introducing flexibility into the enzyme molecule, allowing it to revert to the native structure before imprinting [13]. Thus, the BL enzyme's catalytic behavior was the same as FL's, showing no contribution to hydrolysis degree

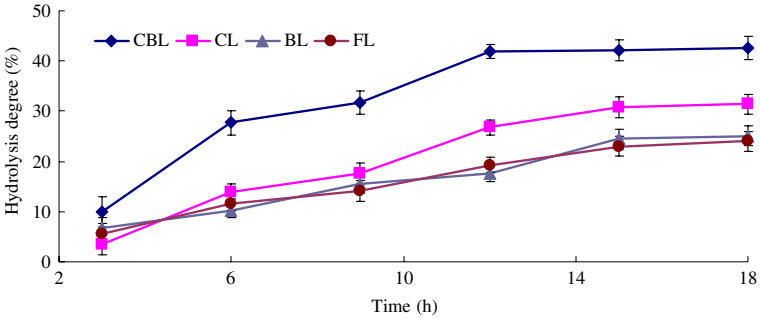

Fig. 1 Preliminary hydrolysis reaction using four enzyme preparations (CBL, CL, BL, and FL). A mixture containing 3 g of crude fish oil, 3 ml of deionized water, and 100 U/(g reaction mixture) of various lipase preparations was incubated at 30 °C with stirring at 300 rpm

enhancement. The crosslinked polymers composed of PEGDA and TMPTA were served as immobilization martrix, and had double effects on these enzyme preparations CL and CBL. Although the network formed by crosslinked polymers might cause mass transfer limitations, the good dispersion of crosslinked enzyme in reaction mixture was also obtained, which provided the opportunity to contact lipase with the substrates more effectively. This could be the reason why CL resulted in higher hydrolysis degree compared to FL. For CBL, although the double effects of crosslinking might also occur, the maintaining of the imprinted properties of enzymes in an aqueous environment was more dominant. Thus, CBL exhibited outstanding hydrolytic activity. It was inferred that BL also possessed the excellent imprinted property prior to contacting the water in reaction mixture, but lost the imprinting effect when subjected to aqueous environment. Palmitic acid was preferably chosen as imprint molecule due to being the main fatty acid constituent present in the original oil (32.7%). When palmitic acid was added to lipase solution, bioimprinting induced conformational changes in the remaining cavity of the enzyme and facilitated matching of substrate functional groups after the imprint molecules were removed. The induced conformation was then trapped by rapid freeze-drying and was stabilized by polymerization in crosslinked polymers. Therefore, CBL exhibited higher catalytic activity compared to other forms (CL, BL, and FL). In the present study, the hydrolysis degree was enhanced significantly by CBL, owing to more removal of non-objective fatty acid constituent ($C_{16:0}$, $C_{16:1}$, and $C_{18:1}$), and higher content of objective fatty acid constituent of EPA and DHA was retained in the glycerides. As shown in Fig. 2, during hydrolysis process by CBL, the content of palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), and oleic acid ($C_{18:1}$) all decreased with increasing the hydrolysis degree, while the content of EPA and DHA increased with increasing the hydrolysis degree. The profile of fatty acid contents in glycerides during the hydrolysis process is similar to that reported by Shimada et al. [19]. Therefore, hydrolysis degree was chosen as an index for evaluation of the enrichment effect of EPA and DHA in further experiments.

Effect of Temperature on Hydrolysis Degree by CBL and FL

Enzymatic hydrolysis reactions were performed at various temperatures by CBL and FL to investigate the effect of crosslinking–bioimprinting modification on enzyme's optimum temperature. As shown in Fig. 3, for FL, hydrolysis degree increased slightly with

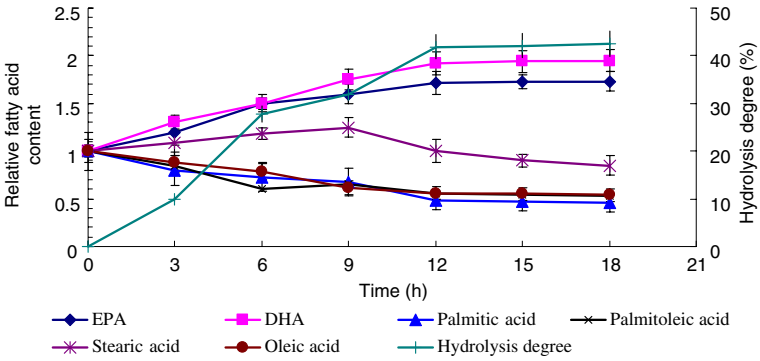

Fig. 2 The main fatty acid contents in glycerides during the preliminary hydrolysis process by CBL. The fatty acid contents in glycerides were expressed as relative contents to those in the original oil

increasing temperature up to 40 °C, and then decreased rapidly until 55 °C, where the inactivation of enzyme occurred. In the case of CBL, it was found that the optimum hydrolysis temperature was 45 °C, and more than 60% of hydrolysis activity remained at 65 °C. The higher optimum hydrolysis temperature and inactivation temperature of CBL suggested that crosslinked network was helpful for maintaining the active structure of the enzyme, and mitigated the formation of unfavorable conformation. The higher optimum hydrolysis temperature and stability in broad temperature range is favorable to bioconversion process compared to the general temperatures of 30–40 °C in lipase-catalyzed reactions [16–20]. In further experiments, temperature of hydrolysis reaction was determined at 40 and 45 °C for FL and CBL, respectively.

Effect of Enzyme Amount on Hydrolysis Degree by CBL and FL

A reaction mixture of 3 g oil, 3 g water, and various amounts of CBL/FL was stirred at 45/40 °C for 12/15 h, to investigate the effect of enzyme amount on the hydrolysis (Fig. 4). The highest hydrolysis degree (25.8%) was achieved when 1.2 mg of FL protein with 600 U was added. Further addition of FL had no more contribution to the hydrolysis

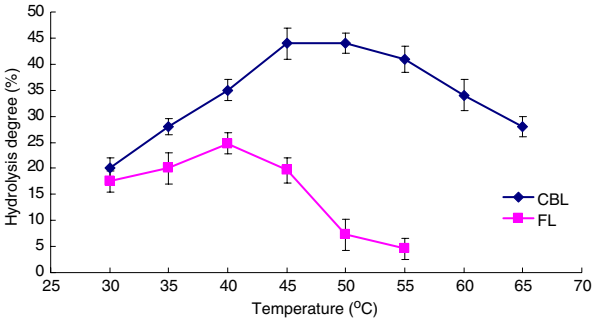

Fig. 3 Effect of temperature on hydrolysis degree by CBL and FL. A mixture containing 3 g of crude fish oil, 3 ml of deionized water, and 100 U/(g reaction mixture) of CBL/FL was incubated at various temperatures with stirring at 300 rpm. The reaction time was 12 and 15 h for CBL and FL, respectively

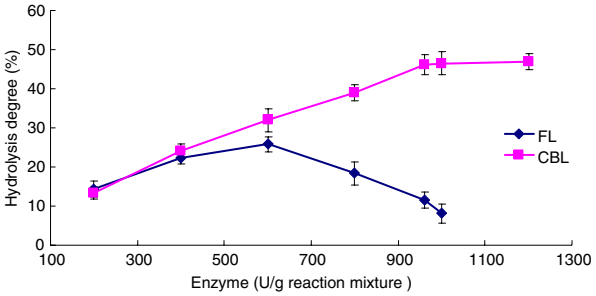

Fig. 4 Effect of enzyme amount on hydrolysis degree by CBL and FL. A mixture containing 3 g of crude fish oil, 3 ml of deionized water, and various amounts of CBL/FL was incubated with stirring at 300 rpm. The reaction times were 12 and 15 h, and reaction temperatures were set at 45 and 40 °C for CBL and FL, respectively

degree. In the case of CBL, reaching the highest hydrolysis degree (46.2%) required only 0.9 mg of enzyme protein with 960 U. The specific activity of CBL was dramatically enhanced compared to that of FL owning to more available accessibility of substrates. FL at higher concentration was more liable to agglomeration. This decreased the actual effective reaction area on the oil–water interface and thus the reaction rate decreased with increasing of lipase concentration [27]. However, CBL in the form of particles contributed to facilitate dispersion of lipase in reaction mixture, thus reducing the agglomeration, so that the reaction rate did not decrease with the increase of lipase concentration.

Time Course of Hydrolysis Reaction by CBL and FL

Based on these results mentioned above, hydrolysis reaction by CBL was performed under the following conditions: a mixture of 3 g crude fish oil, 3 g water, and 0.9 mg of CBL enzyme protein with 960 U was incubated at 45°, with stirring at 300 rpm. For FL, 1.2 mg of FL protein with 600 U was added, and other conditions were the same as mentioned above. Figure 5 shows two typical time courses under optimal conditions. In both of cases, crude oil was hydrolyzed rapidly for several hours after the beginning of the reaction, especially for CBL, and then hydrolyzed more gradually. The required reaction time to

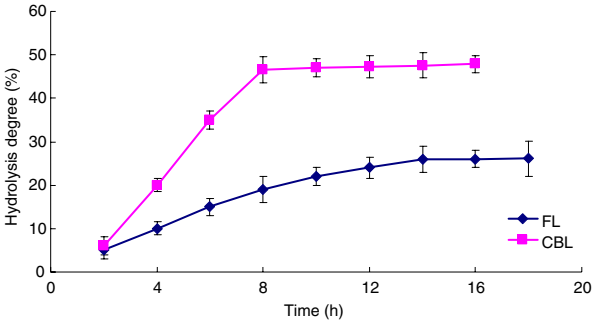

Fig. 5 Time course of hydrolysis reaction by CBL and FL. A mixture containing 3 g of crude fish oil, 3 ml of deionized water was incubated with stirring at 300 rpm. Hydrolysis reaction by CBL was performed at 45 °C and 0.9 mg of enzyme protein with 960 U was added. Hydrolysis reaction by FL was carried out at 40 °C, and 1.2 mg of FL protein with 600 U was added

reach a maximum hydrolysis degree by CBL is 8 h, significantly shorter than that by FL (14 h), which may be attractive from an industrial point of view. Additionally, the overall rate of reaction (8 h) by CBL was 3.1 times that by FL (14 h). Under the optimal hydrolysis conditions for CBL, 46% hydrolysis degree produced glycerides containing 41% of EPA and DHA (EPA+DHA), recovery of 85.7% of initial EPA and DHA (Table 1). There were several publications dealing with enrichment of PUFAs by free preparation or commercially available *Geotrichum candidum* lipase [18, 19, 22]. These differences in hydrolysis degree and PUFAs content can be explained by differences in the oil source and different lipases used, or the same enzyme with different modification.

Operational Stability of CBL and FL

The CBL reusability studies were carried out to determine the enzyme stability in batch reactions. After each run, the enzyme was filtered, washed with water three times, dried at room temperature, and reused. It was found that CBL could retain 80% hydrolysis activity. However, the residual activity of FL was almost not detected in the second reaction batch. CBL subjected to crosslinking of these monomers was structurally more stable in water, withstanding the denaturing conditions much better than corresponding free enzyme.

Conclusions

A combined method composed of crosslinking and bioimprinting was introduced to modify *Geotrichum* sp. lipase for use in aqueous hydrolysis reaction. Comparison of crosslinked lipase, bioimprinted lipase, crosslinked bioimprinted lipase, and free lipase suggested that crosslinked polymerization not only stabilized imprinted enzyme property, but also contributed to facilitate dispersion of lipase in reaction mixture, thus reducing the agglomeration. The resulting crosslinked bioimprinted lipase showed higher hydrolysis temperature, enhanced specific activity and shorter hydrolysis time compared to free lipase. Moreover, CBL was reused for five cycles without a significant loss of activity, showing a greater stability than free enzyme. Selective hydrolysis of crude fish oil based on the CBL biocatalyst showed that hydrolysis degree as well as over rate of reaction increased compared to free lipase. Under the following conditions: a mixture containing 3 g of crude fish oil, 3 ml of deionized water, and 0.9 mg of enzyme protein with 960 U incubated at 45 °C with stirring at 300 rpm for 8 h, 46% hydrolysis of crude fish oil produced glycerides containing 41% of EPA and DHA (EPA+DHA), achieving 85.7% recovery of initial EPA and DHA.

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