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Investigation of synthetic lipase and its use in transesterification reactions

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ABSTRACT

A novel synthetic polymer selective for p-nitrophenylpalmitate was synthesized by molecular imprinting technique. We have combined the principle of molecular imprinting with the ability of histidine, glutamic acid and serine to form a catalytic cavity that can promote the catalytic degradation of p-nitrophenyl palmitate.

For the creation of such catalytic sites we first synthesized appropriate monomers and used p-nitrophenyl palmitate as a template to synthesize the imprinted polymers and the binding characteristics of the polymers were evaluated. The optimum pH was determined by evaluating different pH values and the hydrolytic activity of synthetic lipase was evaluated in the framework of Micheaelis–Menten kinetics. In addition, the values of maximal rate: Vm (0.68 mM/min) and Michaelis–Menten constant, Km (1.4×10^{-2} mM) were obtained from Lineweaver–Burk plots for the imprinted polymeric catalyst.

1. Introduction

Molecular imprinting is a method for making selective binding sites in synthetic polymers by using a molecular template. Target molecules can be used as templates for imprinting crosslinked polymers. After the removal of the template, the remaining polymer is more selective. The selectivity of the polymer depends on various factors like the size and the shape of the cavity and rebinding interactions. Covalent interactions [1–4], non-covalent interactions [5–9], electrostatic interactions [10] and metal ion coordination [11–13] can be exploited to organise the functional monomers around the template.

The molecularly imprinted polymers (MIPs) have been used for separations, diagnostic assays, biosensors and biocatalysis. MIPs have also been employed in producing of synthetic enzyme catalysis applications. A detailed review about this subject [14] and some applications of MIPs in catalysis [15–27] have been reported.

Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases and therefore do not require any cofactor. The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyse them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids [28], a wide variety of reactions, such as hydrolysis, transesterification, and aldol reactions. However, one of the problems of using enzymes as catalysts is their serious limitations in terms of recovery [29], when enhancing the stability and maintaining the activity of the biocatalyst, the choice of the enzyme support is of extreme importance [30,31]. Lipases have histidine–glutamic acid and serine trial amino acid group in the active centre.

On the other hand, Methyl jasmonate (MeJA) is a substance used in plant defence [32] and also under early research for cancer treatment in humans. Plants produce jasmonic acid and methyl jasmonate in response to many biotic and abiotic stresses (particularly herbivory and wounding), which build up in the damaged parts of the plant.

In this study, we have used advantages of high selectivity of molecular imprinting technique for synthesis of artificial lipase for catalysis of p-nitrophenyl palmitate and using in synthesis of biodiesel and methyl jasmonate.

2. Experimental section

2.1. Chemicals

Candida Rugosa lipase and p-nitrophenyl palmitate were purchased from Sigma (St Louis, MO, USA). Ethyleneglycol dimethacrylate (EDMA) was purchased from Fluka A.G. (Buchs, Switzerland), distilled under reduced pressure in the presence of hydroquinone inhibitor and stored at 4 °C until use. Azobisisobutyronitrile (AIBN) and Poly (vinyl alcohol) (PVA; MW: 22,000) were purchased from Aldrich Chem. Co. (Milwaukee, WI, USA). All

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glassware was extensively washed with diluted nitric acid before use. Water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANO pure® organic/colloid removal and ion exchange packed-bed system.

2.2. Synthesis of p-nitrophenyl palmitate imprinted polymeric beads

The MASE, MAH, and MAGA monomers were synthesized according to previously published procedure [33].

An appropriate amino acid (5.52 mmol, 1 g, 1 eq.) was dissolved in 1 M aqueous solution of NaOH (1 eq.) in a round bottom-flask. A solution of MA-Bt (5, 52 mmol, 1.033 g, 1eq) in 25 mL of 1,4-dioxane was slowly added into the amino acid solution. The reaction mixture was allowed to stir for 10–20 min at room temperature. Completion of the reaction was monitored by TLC, after the reaction finished, 1,4-dioxane was evaporated under vacuum. The residue was diluted with water and extracted with ethyl acetate (3 × 50 mL) to remove 1 *H*- benzotriazole. Collected water phases were neutralized to pH = 6–7 using 10% water solution of HCl (pH should keep around 6–7 to prevent possible polymerization of methacryloyl group in acidic medium). Then, water was removed *via* rotary-evaporator to give amino acid monomer in 85–90% yield.

P-nitrophenyl palmitate imprinted beads (PIBs) were synthesized by suspension technique as follows. The dispersion medium was prepared by dissolving 0.15 g of poly(vinyl alcohol) in 60 mL of distilled water. Methacryloylamido serine (MASE), methacryloylamido histidine (MAH) and methacryloylamido glutamic acid (MAGA) monomers (0.1 mmol) and p-nitrophenyl palmitate (0.1 mmol) as a template molecule were added into 6.0 mL of EDMA/12.0 mL of toluene–acetonitrile mixture (80:20); then 0.609 mmol of 2,2-azobisisobutyronitrile (AIBN) was dissolved in the monomer mixture. This solution was then transferred into the dispersion medium placed in a magnetically stirred (at a constant stirring rate of 600 rpm) glass polymerization reactor (100 mL) in a thermostatic water bath. The reactor was flushed by bubbling nitrogen and then was sealed. The reactor temperature was kept constant at 75 °C for 8 h. Then the polymerization was completed at 90 °C during 4 h. After polymerization, the PIBs were separated from the polymerization medium. The template was removed by washing procedure using 100 mL of 50/50 v/v methanol/water containing 1.0 M of KOH for 24 h. In Fig. 1, Schematic representation of p-nitrophenyl palmitate (pNPP)-template reconstruction in polymeric structure is given.

2.3. Activity assay of synthetic lipase

Lipase activity was measured by the spectrometric method using *p*-nitrophenyl palmitate (*p*-NPP) as a substrate. The activity was assayed by measuring the absorbance of liberated *p*-nitrophenol at 405 nm [34].

2.4. Biodiesel production and jasmonic acid esterification by using synthetic lipase

2.4.1. Transesterification reactions

Transesterification reactions were carried out in 50 ml reactors containing a mixture of 5 g of olive oil, 6 g of methanol and 0.5% water (w/w) using 25 mg of synthetic lipase for biodiesel production. The pH of reaction medium was adjusted to 8.0 with methanolic NaOH. The reaction mixture was incubated at 50 °C and shaken in an reactor thermomixer. In the second step of the experiments, it was used 14 mg of jasmonic acid and 14 mL of

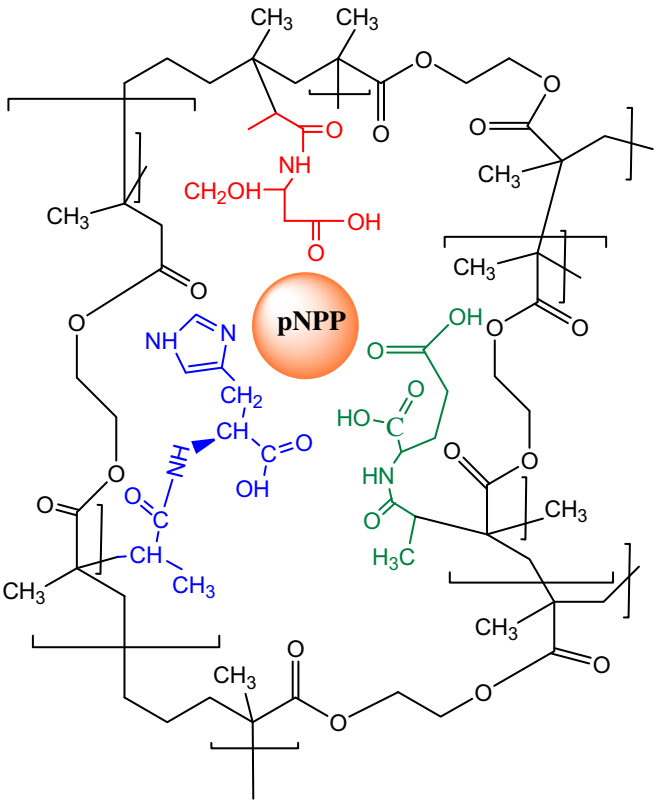

Fig. 1. Schematic representation of p-nitrophenyl palmitate (pNPP)-template reconstruction in polymeric structure.

methanol were used for esterification using the same amount of synthetic catalyst. After each batch reaction, the synthetic catalyst was recovered by filtration, washed with distilled water and dried at room temperature. Dried synthetic catalyst was used in the next batch reaction for new substrates. This proves that this catalyst can be used repeatedly.

2.4.2. GC–MS analysis

GC MS analysis was performed by injecting 1 μL of reaction mixture into a Shimadzu GCMS-QP5050 instrument equipped with an apolar column (25 × 0.25 mm id, CP Sil 5 CB). Analysis was carried out using gradient temperature program running from 60 °C to 180 °C at 2 °C/min and 180 °C to 240 °C at 2 °C/min. Injection and detection temperature were fixed at 250 °C using helium as carrier gas.

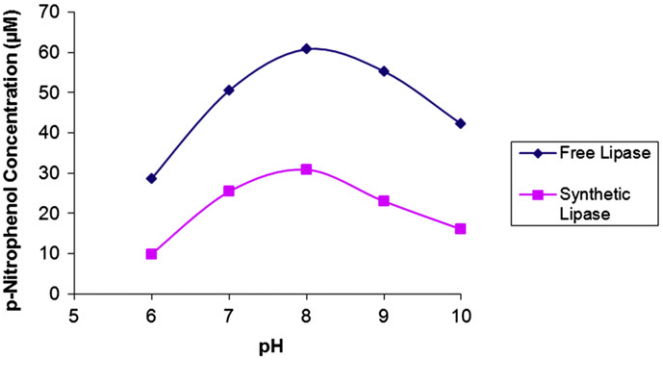

Fig. 2. Effect of pH on p-NPP hydrolysis using free and synthetic lipase.

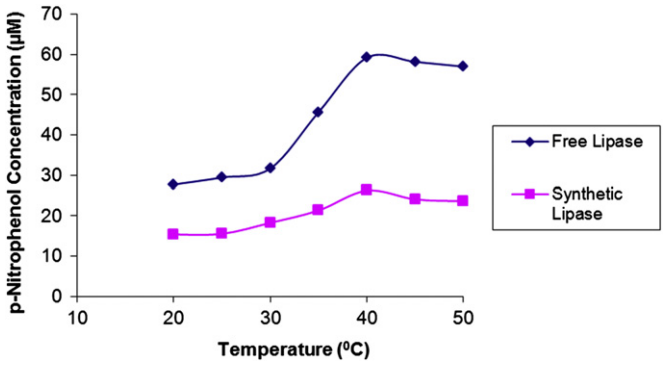

Fig. 3. Effect of temperature on p-NPP hydrolysis using free and synthetic lipase.

3. Results and discussion

3.1. Effect of pH on p-nitrophenyl palmitate (p-NPP) hydrolysis

The pH is one of the important parameters that can affect the enzymatic activity in aqueous solution. In this study, the optimum pH was determined by evaluating different pH values in the range of 6.0–10.0 using 20 mM p-nitrophenylpalmitate as a substrate. The effect of pH on the time course of p-nitrophenylpalmitate hydrolysis is shown in Fig. 2. As seen from the figure, the maximum hydrolytic activity was observed at pH 8.0.

3.2. Effect of temperature on p-nitrophenyl palmitate (p-NPP) hydrolysis

It is known that temperature has a strong influence on the catalytic activity of a catalyst. Here we have evaluated a temperature range between 20 °C–50 °C. Fig. 3 shows the effect of temperature on p-nitrophenylpalmitate hydrolysis.

As can be seen from the figure, the optimum temperature is at approximately 40 °C.

3.3. Hydrolytic activity of synthetic lipase

Fig. 4 shows the time course of p-nitrophenyl palmitate hydrolysis by the synthetic lipase. The kinetic constants were obtained by fitting the data to equation (1), where V is the initial rate, Vm is the maximum rate, S is the concentration of substrate, Km is the Michaelis-Menten constant.

$$V = Vm \cdot S/(Km + S) \quad (1)$$

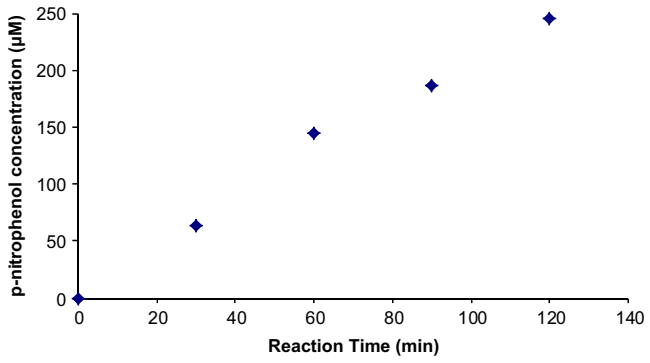

Fig. 4. p-Nitrophenyl palmitate hydrolysis using synthetic lipase at pH 8.0.

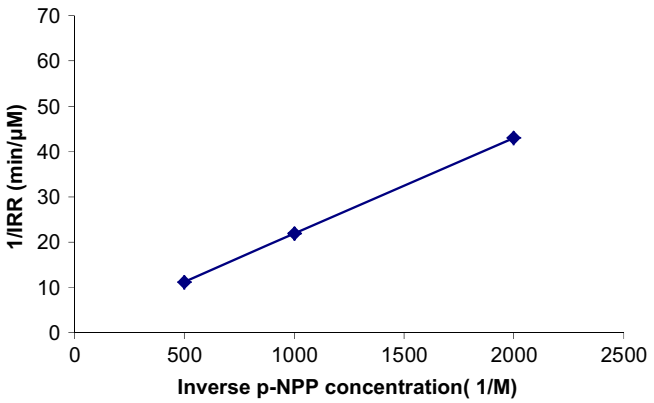

Fig. 5. Lineweaver–Burk plot of kinetics data of hydrolysis of paranitrophenyl palmitate.

The hydrolytic activity of synthetic lipase was evaluated with respect to Michaelis–Menten formula and kinetics. For this purpose, p-nitrophenyl palmitate substrate concentrations were varied from 2 mM to 20 mM and initial reaction rates (IRR) of hydrolysis were determined. Then, 1/IRR versus reciprocal of the substrate concentration (1/S) for polymeric beads was plotted which is also known as the Lineweaver–Burk plot (Fig. 5) and the values of Vm and Km were obtained from plots as 0.68 mM/min and $1{,}4 \times 10^{-2}$ mM, respectively.

In Michaelis–Menten kinetics, Km reflects the affinity of an enzyme for a particular substrate i.e. the lower value of Km the higher affinity [17]. In the present case Km represents the affinity of functional groups of mimics lipase for substrate p-nitrophenyl palmitate in the imprinted material.

3.4. Biodiesel production and jasmonic acid esterification by using synthetic lipase

In this part of the study, we wanted to evaluate the synthetic lipase to use in transesterification reactions. The fatty acid methyl ester and methyl jasmonate conversion are seen in Fig. 6.

FAME conversion of fatty acid in olive oil is 92.97% and the conversion of jasmonic acid to methyl jasmonate by using synthetic lipase is 100%.

These results show that synthesized biomimetic artificial enzyme can effectively be used in transesterifaction reactions. The main advantages of synthetic lipase used in this work are high thermal and chemical stability, low cost and reusability compared to natural lipase.

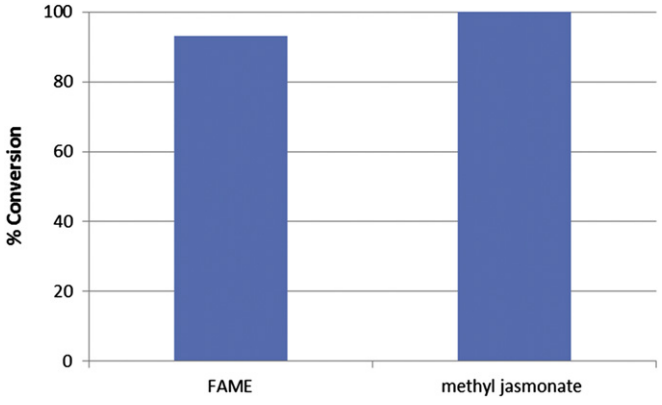

Fig. 6. Conversion of fatty acids and jasmonic acid using synthetic lipase.

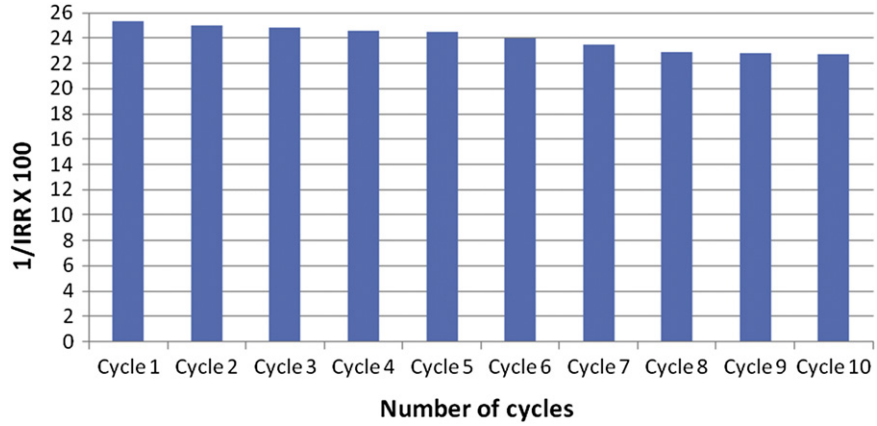

Fig. 7. Reusability of synthetic lipase.

3.5. Reusability of synthetic lipase

In order to show the stability and reusability of the lipase mimic polymer particles, the experimental cycle was repeated 10 times using the same particles. For sterilization after one cycle, the particles were washed with 1 M NaOH solution and then the particles were washed with distilled water.

As seen in Fig. 7, the particles are very stable even after 10 cycles.

4. Conclusions

In this study we have designed and synthesized a new type of imprinted material with a catalytic activity. The material was prepared using MAH, MAGA and MASE monomers. Those were chosen with the purpose to improve the stability, decrease the cost and to yield a selective catalyst towards p-nitrophenyl palmitate hydrolysis and esterification reactions. The preparation of the polymer is simple and straight forward. The results demonstrate that the catalytic activity of microbeads has decreased by only 9% after 10 uses Thus, the imprinted polymer developed in this study can be used as an efficient mimic catalyst for the hydrolysis of p-nitrophenyl palmitate.

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