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ARTICLE *in* BIOTECHNOLOGY AND BIOPROCESS ENGINEERING · NOVEMBER 2014

Impact Factor: 1.11 · DOI: 10.1007/s12257-014-0015-7

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Biotechnology and Bioprocess Engineering 19: 892-899 (2014)
DOI 10.1007/s12257-014-0015-7

RESEARCH PAPER

Lipase Purification by Affinity Precipitation with a Thermo-responsive Polymer Immobilized Cibacron Blue F3GA Ligand

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Received: 6 January 2014 / Revised: 11 May 2014 / Accepted: 16 May 2014

Abstract A thermo-responsive polymer (P_{NNB}) was synthesized with lower critical solution temperature 27.5ºC and over 95% recovery. The adsorption of porcine pancreatic lipase on Cibacron Blue F3GA-conjugated P_{NNB} (P_{NNB}-CB) closely followed the bi-Langmuir adsorption isotherm. The maximum adsorption capacity was found at pH 5.0, with a ligand density of 18.4 μmol/g polymers. The optimized eluent was a 0.01 M phosphate buffer solution at pH 8.0 containing 20% ethylene glycol. Six adsorption-desorption recycles indicated excellent reusability of the affinity adsorbent. P_{NNB}-CB was applied to separate porcine pancreatic lipase from its crude material giving a lipase activity recovery of 81.6% with a 16-fold purification factor. Lipase could be purified to single-band purity, according to gel electrophoresis. The purification strategy is therefore feasible and efficient for purifying proteins of interest.

Keywords: affinity precipitation, thermo-responsive polymer, lipase, cibacron blue F3GA

1. Introduction

Lipases catalyze not only the hydrolysis of fats into fatty acids and glycerol in the aqueous phase but also esterification, inter-esterification and trans-esterification reactions in organic media. Because of their applications in detergent formulations, nutrition, leather, cosmetics, and pharmaceuticals, lipases now comprise a significant proportion of biocatalysts [1,2]. Traditionally, most purification processes for lipases comprise a precipitation step followed by a combination of several chromatographic steps such as ion exchange chromatography, gel filtration, and affinity chromatography [3,4]. The main drawbacks of traditional purification methods include low yields, with multiple steps and long process times [5]. Several new, alternative purification methods, including interfacial affinity chromatography [6,7], specific lipase-lipase interactions [8], lipase-specific separation-based affinity chromatography [9], and aqueous two-phase systems [10,11] have been developed to purify lipases.

Over the last few decades, many studies have focused on affinity precipitation technology. Numerous enzymes [12,13], proteins [14], polysaccharides [15], and DNA [16,17] have been purified by affinity precipitation. Affinity precipitation is a promising bio-separation method with high selectivity, achieved by soluble-insoluble polymers that respond reversibly through application of heat, pH, or special ions. Amongst these polymers, thermo-[18] and pH-responsive polymers [19] are usually used in bio-separation. Desirable responsive polymers should have excellent reusability and a suitable lower critical solution temperature (LCST) or isoelectric point. Compared with affinity precipitation induced by other conditions, thermo-affinity precipitation requires only the input of energy, rather than matter, into the system, leading to a minimum of waste and contamination [20]. An ideal thermo-responsive polymer should be rationally designed for application to the purification of proteins [21]. Dye ligands play an important role in affinity separation technology because of their low cost, good stability, and group specificity. A large number of proteins and enzymes have been separated by dye ligands [22,23]. In particular, Cibacrion Blue F3GA (CB) is frequently

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applied to purify proteins. Denizli [24] purified lysozyme with CB-affinity beads under a magnetic field. Ding [19] separated cellulase by pH-responsive affinity precipitation with a CB ligand.

A thermo-responsive polymer (P_{NNB}) was synthesized and used to purify lysozyme from eggs in a previous report [25], However, the recovery of the polymer was only 85.0% in aqueous solution. Lipase was purified in our group *via* affinity precipitation with a butyl ligand [26]. Acetone and boron trifluoride were used to immobilize the ligand, which may result in a pollution risk and potential safety hazards during a enlarging scale-up.

In this study, we modified the method for synthesizing the thermo-responsive polymer P_{NNB} by optimizing the molar ratio of the three monomers involved in the polymerization. CB was readily immobilized onto the polymer as the affinity ligand. The optimal adsorption and elution conditions for lipase on the CB-conjugated polymer (P_{NNB}-CB) were studied. Finally, P_{NNB}-CB was applied to separate lipase from its crude source material with a number of interesting results. It is a new report that the dye ligand Cibacron Blue F3GA has been used to purify lipase.

2. Materials and Methods

2.1. Materials

Cibacron Blue F3GA (CB) and bovine serum albumin were purchased from Sigma (MO, America). Porcine pancreatic lipase powder was kindly supplied by ADFN Biotechnology Co., Ltd (Nanjing, China). N-isopropyl acrylamide (NIPAM), butyl acrylate (BA), azobis-isobutyronitrile (AIBN), and N-hydroxymethyl acrylamide (NHMA) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All other chemicals and reagents used were of reagent grade.

2.2. Experimental methods

2.2.1. Synthesis of P_{NNB}

The method for synthesizing P_{NNB} was based on a previously described method [25] by random polymerization with several modifications. Specified amounts of monomers including NIPAM, NHMA, and BA were poured into a flask containing 100 mL ethanol as a solvent and 50 mg AIBN as the polymerization initiator. A nitrogen atmosphere was then applied for 10 min to remove oxygen. The reaction was carried out for 24 h at 60ºC in a constant temperature water bath and ethanol then removed by distillation. The residue was dissolved in 10 mL acetone and then precipitated in 200 mL hexane. Finally, the precipitate was collected and dried in a vacuum drying oven.

2.2.2. Preparation of P_{NNB}-CB

The immobilization of CB on P_{NNB} was performed according to a method previously described by our group [25]. One hundred milligrams of CB and 1.0 g P_{NNB} were dissolved in 50 mL of distilled water, then 1.0 g NaCl was added to the solution. The reaction was maintained at 40ºC in a water bath for 1 h, and then 1.5 g Na_2CO_3 was added to adjust the pH of the solution to 11.5. This reaction was carried out for 24 h at 60ºC. After the reaction reached equilibrium, the polymer was washed thoroughly with distilled water and a 20% ethanol solution to remove unbound dye. The remaining CB solution and all washing solutions were collected and diluted to 500.0 mL and the absorbance measured at 610.0 nm with a Shimadzu UV mini-1240 UV-VIS spectrophotometer (Tokyo, Japan). CB concentration was calculated using a standard calibration established with 0.01 ~ 0.1 mg/mL CB solutions. Ligand density (μmol/g polymer) on P_{NNB} was calculated by CB mass balance between the initial and residual solution.

2.2.3. Adsorption of lipase on P_{NNB}-CB

P_{NNB}-CB was dissolved in 10 mL lipase solution (pH 7.2, 0.2 mg/mL, 344 U/mg protein), and the mixture was incubated in a shaking water bath at 20®¨C and 120 rpm for 2 h. The solution was then heated to the LCST to precipitate the lipase-P_{NNB}-CB complex and centrifuged at 2,180 g and 35.0°C for 5 min. The activity of lipase in the supernatant was measured and the amount of lipase bound on the P_{NNB}-CB was determined by mass balance after measuring the initial and residual concentrations of lipase by the Bradford method [27]. Meanwhile, 0.1 g P_{NNB} was used as a control experiment using the same steps as above. Adsorption conditions were optimized by varying the free lipase concentration (0.0 ~ 0.7 mg/mL), CB density (0.0 ~ 22.0 μmol/g), pH (4.0 ~ 9.0 buffers) and ionic strength (0.0 ~ 0.5 mol/L NaCl).

2.2.4. Desorption of bound lipase

The collected precipitates were re-dissolved in 10 mL of different eluents (1.0 mol/L NaCl, 1.0 mol/L KSCN, 20% ethylene glycol containing 1.0 mol/L NaCl, and 20% ethylene glycol in 0.01 mol/L phosphate buffer solution at pH 8.0) and incubated in a water bath at 20ºC and 120 rpm for 2 h. The solution was subsequently heated to the LCST to precipitate P_{NNB}-CB. The amount and activity of the lipase desorbed from P_{NNB}-CB was determined by the aforementioned methods.

2.2.5. Reusability of P_{NNB}-CB

After lipase desorption experiments were completed, the

P_{NNB}-CB was treated with 1.0 M HCl for 1 h to completely remove residual contaminants. The recovered P_{NNB}-CB was then dried, weighed, and reused in the subsequent cycle of affinity precipitation.

2.2.6. Application of P_{NNB}-CB to purification of lipase

Affinity precipitation of lipase from crude material was carried out by the process described above. Crude lipase solution consisted of crude porcine pancreatic lipase in 0.02 mol/L pH 7.0 phosphate buffers, and the activity of the solution was 21.0 U/mg. The purified lipase was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and its specific activity was also determined.

2.3. Analytical methods

2.3.1. Determination of LCST and recovery

Cloud point measurements were performed by immersing a test tube containing aqueous P_{NNB} solutions in a water bath heated with a temperature increase rate of 0.5°C/min. A 1 cm sample cell was used and the temperature was set between 22 and 35°C. Concentrations of solutions were in the range of 0.1 ~ 10% wt and were analyzed using a Shimadzu UVmini-1240 UV-VIS spectrophotometer. Cloud points were defined as the temperature corresponding to a 10% reduction in the original transmittance of the solution [28]. LCST was identified as the lowest point in the cloud points curve. The recovery of the polymer was determined as the ratio of the dried weight of the precipitated polymer recovered by heating compared with that of the initial weight.

2.3.2. Activity assay of lipase

The activity of lipase was determined by olive oil hydrolysis [2]. One lipase unit is defined as that which releases 1.0 μmol of fatty acid per minute under the assay conditions. The specific activity is activity units per milligram protein.

3. Results and Discussion

3.1. Synthesis of P_{NNB}

The molar ratio of three monomers during polymerization has a great impact on the properties of the resultant thermo-responsive polymers. In this study, the molar ratio of NHMA, NIPAM, and BA were modified from 7:14:3.5, respectively, reported in previous research [25] to 1:14:1.5. The recovery of P_{NNB} was 95.0% and the LCST was 27.5ºC, compared with 83.5% and 28.0ºC as reported previously [25].

Amongst the three monomers, NIPAM provides the thermo-responsive character of the polymer; NHMA provides hydroxyl group in the polymer for immobilization of affinity ligands and controlling the solubility of the polymer; while the hydrophobic monomer BA helps to control the LCST as well as recovery of the polymer. An increase in the ratio between BA and NHMA can reduce the LCST of the polymer when NIPAM remains constant. However, it may also enhance the recovery of the polymers, likely attributable to strengthened hydrophobic interactions. Considering the conditions in the subsequent experiments and the influence of temperature on the stability of general biomolecules, a LCST of 27.5ºC is an ideal temperature in a pure aqueous solution. An excellent polymer recovery was also achieved with this polymer enabling a greater number of recycles for large-scale application.

3.2. Influence of ionic strength on P_{NNB}

Generally, ionic strength may affect the solubility of thermo-responsive polymers [29]. Fig. 1 shows the effect of NaCl concentration on LCST and recovery of P_{NNB}. The LCST declined and the recovery clearly increased with NaCl concentration increasing from 0.0 to 0.5 mol/L. The recovery of the polymer was almost 100%, with an LCST of 19.5ºC in the presence of 0.5 mol/L NaCl. To precipitate completely P_{NNB} in subsequent experiments under different conditions, 27.5ºC was determined as the LCST.

This interesting observation can be explained by the role played by hydrogen bonds in solubility and precipitation of thermo-responsive polymers. It is well known that hydrogen bonds provide the principal interactions controlling the solubility of thermo-responsive polymers. The weakening of hydrogen bonds could result in higher hydrophobicity and contribute to the aggregation and precipitation of

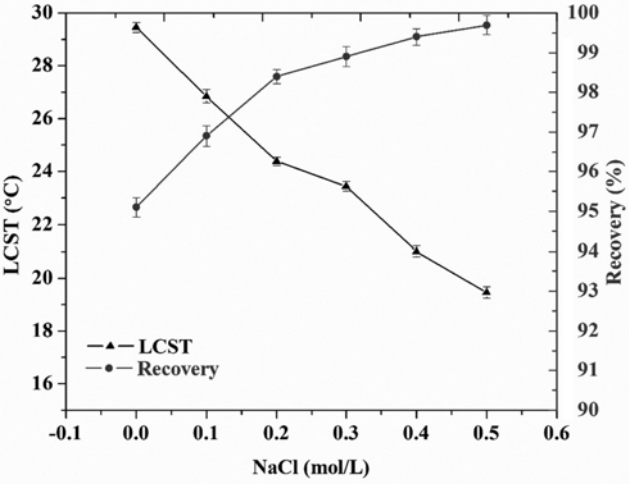

Fig. 1. The effect of NaCl concentration on the LCST and recovery of polymer P_{NNB}.

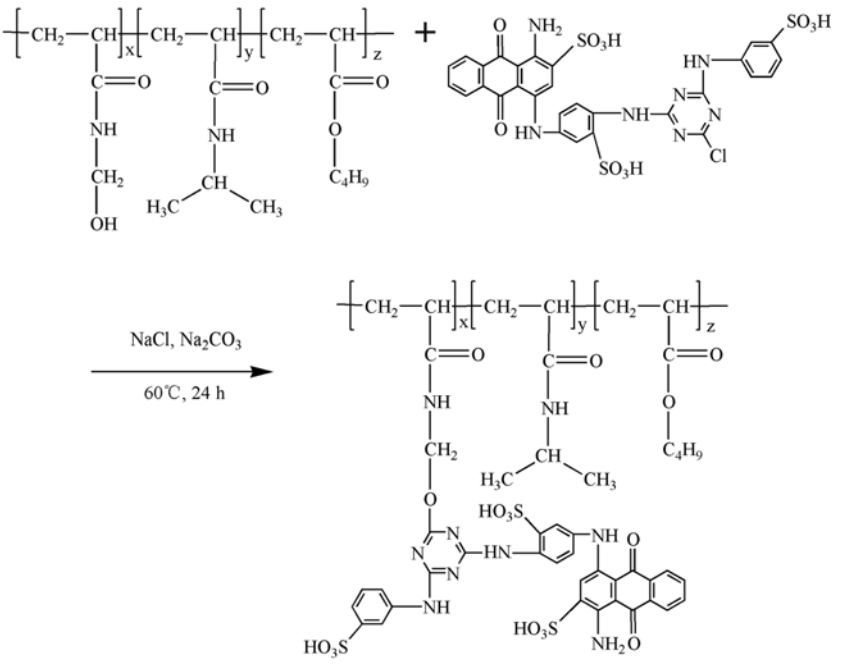

Fig. 2. The process for preparing P_{NNB}-CB. Reaction conditions: 340 mmol/L NaCl, 280 mmol/L Na_2CO_3, 60°C, 24 h.

the thermo-responsive polymer at increased temperature [30,31]. Sodium and chloride ions could compete with the polymer in combining with water molecules to weaken the hydration of hydrophobic groups in the polymer chain. Consequently, the polymer tends to precipitate at lower temperature and give a higher recovery in the presence of NaCl.

3.3. The immobilization of CB on P_{NNB}

The process of preparing P_{NNB}-CB is illustrated in Fig. 2. CB can be readily immobilized on the polymer *via* the nucleophilic reaction between the active chlorine atoms in the triazine rings and hydroxyl groups on the polymer. Additionally, the coupling process is environmentally friendly.

The LCST and recovery of P_{NNB}-CB were almost the same as for the native polymer P_{NNB}. As for the native polymer, the addition of NaCl caused a decrease in the LCST and a higher recovery of P_{NNB}-CB as well.

3.4. Adsorption of lipase on P_{NNB}-CB

3.4.1. Adsorption kinetics curve

As shown in Fig. 3A, the amount of lipase bound on P_{NNB} increased with time, reaching equilibrium at 30 min and the binding capacity was 2.3 mg/g. The amount of lipase absorbed on P_{NNB}-CB increased significantly, reaching equilibrium at 40 min and the adsorption capacity was 8.7 mg/g. In subsequent experiments, adsorption for 2 h was carried out to guarantee stable binding between P_{NNB}-CB and lipase.

3.4.2. Effect of CB density on lipase binding

The effect of CB density on lipase adsorption is shown in Fig. 3B. It is shown that the amount of lipase binding on P_{NNB}-CB was strongly dependent on the density of CB on the polymer. The amount of lipase binding increased with CB density from 0.0 to 22.1 μmol/g polymer and the adsorption capacity for lipase reached 9.0 mg/g when the ligand density was 22.1 μmol/g. On the contrary, the amount of lipase bound on P_{NNB} was only 2.2 mg/g (zero CB density in Fig. 3B), indicating that adsorption capacity of lipase was mainly attributed to specific interactions between CB molecules and lipase molecules. However, the relationship between adsorption capacity and CB density was not a linear dependence. The lipase adsorption capacity was almost constant when CB density was exceeded 18.4 μmol/g polymer. The reasons for this were a gradual increase in steric effects such as binding site blockage by adsorbed lipase molecules or electrostatic repulsion between negatively charged P_{NNB}-CB and lipase with the same charge.

3.4.3. Effect of ionic strength on lipase adsorption

The effect of ionic strength on lipase adsorption is shown in Fig. 3C. The amount of lipase bound on P_{NNB} increased slightly from 2.1 to 2.6 mg/g, whereas the adsorption capacity for lipase on P_{NNB}-CB significantly increased from 8.4 to

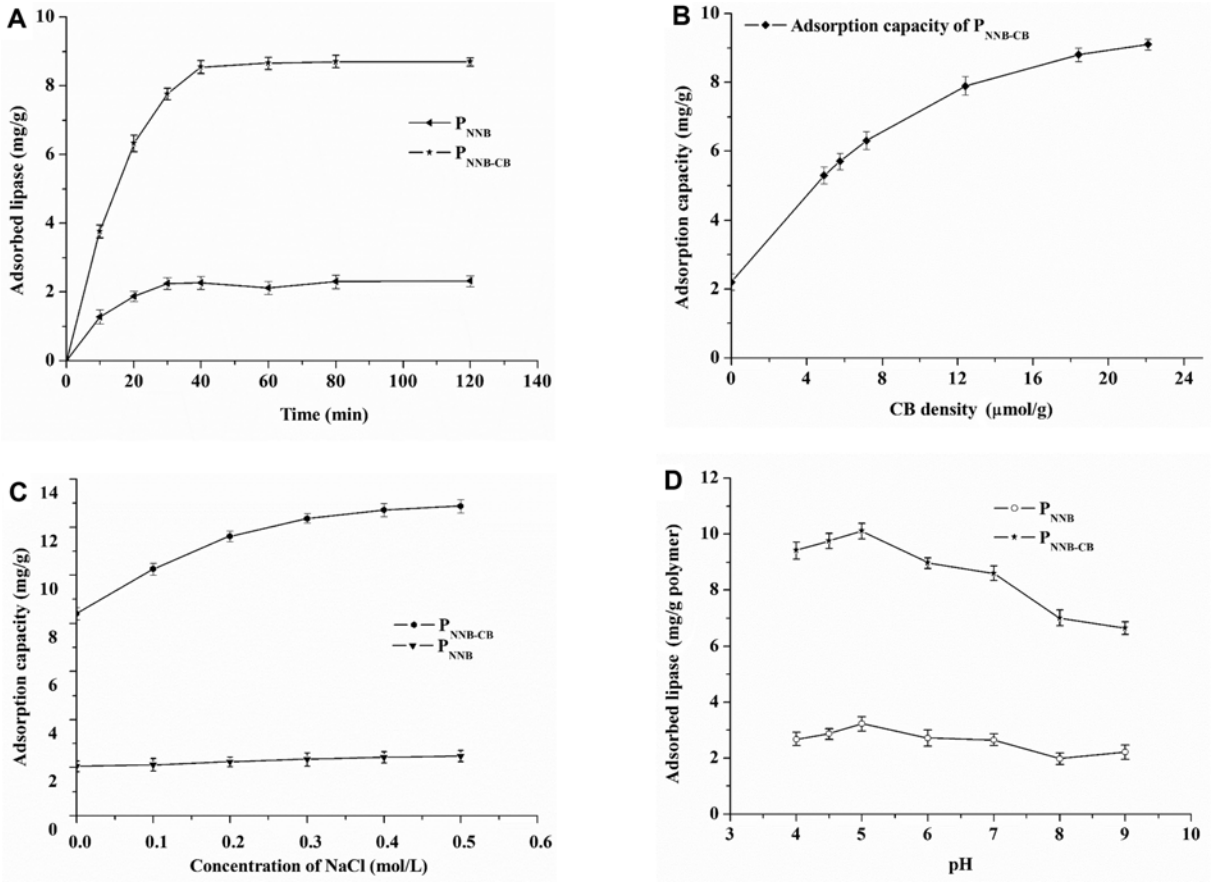

Fig. 3. (A) Lipase adsorption kinetics. Reaction conditions: 0.1 g control P_{NNB} or P_{NNB}-CB with a CB density of 18.4 μmol/g, 10 mL of 0.2 mg/mL lipase, pH 7.2, 20°C, and 120 rpm shaking for 2 h. (B) Effect of CB density on lipase adsorption. Reaction conditions: 0.1 g P_{NNB}-CB with different CB densities, 10 mL of 0.2 mg/mL lipase, pH 7.2, 20°C, and 120 rpm shaking for 2 h. (C) Effect of NaCl concentration on lipase adsorption. Reaction conditions: 0.1 g control P_{NNB} or P_{NNB}-CB with a CB density of 18.4 μmol/g, 10 mL of 0.2 mg/mL lipase, pH 7.2, 20°C, and 120 rpm shaking for 2 h. (D) Effect of pH on lipase adsorption. Reaction conditions: 0.1 g P_{NNB} or P_{NNB}-CB with a CB density of 18.4 μmol/g, 10 mL of 0.2 mg/mL lipase, 20°C, and 120 rpm shaking for 2 h.

12.8 mg/g with an increasing NaCl concentration from 0.0 to 0.5 mol/L. Sodium ions could weaken the electrostatic repulsion forces between lipase molecules and P_{NNB}-CB molecules by neutralizing the negative charges.

3.4.4. Effect of pH on lipase adsorption

Fig. 3D shows that the maximum adsorption capacity appeared at pH 5.0, which is the isoelectric point of porcine pancreatic lipase. A higher lipase adsorption capacity on P_{NNB}-CB was found to be at pH 4.0 ~ 5.0, but decreased significantly when the pH increased from 5.0 to 9.0.

Generally, protein-CB binding can be attributed to electrostatic and hydrophobic interactions [32]. The highest lipase adsorption capacity was obtained at pH 5.0 (the pI of lipase) because of weak electrostatic repulsion and strong hydrophobic interactions. When the pH of the solution was below 5.0, the sulfonate groups of CB molecules became negatively charged and the lipase molecules positively charged. Consequently, a high adsorption capacity was obtained through the intense electrostatic attraction between the molecules at pH 4.0 ~ 5.0. Results show the adsorption of lipase was slightly lower when the pH was below 4, indicating that electrostatic repulsions between adjacent adsorbed lipase molecules. When the solution pH was above the pI of lipase, the decline in the lipase adsorption capacity was caused by the electrostatic repulsion between negatively charged lipase molecules and CB molecules of the same charge. This section demonstrates that the pH of the solutions had a great effect on adsorption.

3.4.5. Adsorption isotherm

Fig. 4 shows the adsorption isotherms for lipase binding on P_{NNB} and P_{NNB}-CB, both of which could absorb lipase. The question arises as to how lipase bound on the control polymer P_{NNB}. Lipase has a higher hydrophobicity than conventional proteins and an inherent affinity toward

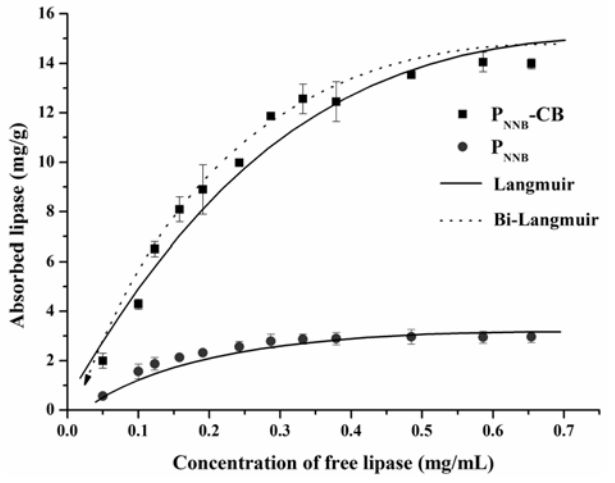

Fig. 4. Lipase adsorption isotherm. Reaction conditions: 0.1 g control P_{NNB} or P_{NNB}-CB with a CB density of 18.4 μmol/g, 10 mL lipase, pH 5.0, 20°C, and 120 rpm shaking for 2 h.

hydrophobic media through hydrophobic interactions. Some literatures reports describe direct that lipase immobilization or purification using hydrophobic supports such as butyl-, phenyl-, or octyl-agarose and multi-walled carbon nanotubes [7,9,33]. P_{NNB} contains butyl groups, so it is hydrophobic to some extent. A low amount of lipase could therefore be adsorbed onto P_{NNB} *via* hydrophobic interactions. The markedly enhanced lipase adsorption capacity on P_{NNB}-CB compared with the control polymer P_{NNB}, can be attributed to the presence of CB ligand molecules.

The Langmuir (Equation 1) and bi-Langmuir (Equation 2) equations were chosen to describe the adsorption isotherm of lipase:

$$q = \frac{q_0 C}{K_d + C} \quad (1)$$

$$q = \frac{q_1 C}{K_{d1} + C} + \frac{q_2 C}{K_{d2} + C} \quad (2)$$

where C and q are the free concentration of lipase in solution and adsorbed onto the solid phase, respectively; q_0, q_1, and q_2 are the maximum adsorption capacities, and K_d, K_{d1}, and K_{d2} are the dissociation constants.

As presented in Fig. 4 and Table 1, the Langmuir fitting results show that the adsorption capacity of lipase on P_{NNB}-CB was much higher than on P_{NNB} and the adsorption of lipase on P_{NNB}-CB was more stable. However, it was found that fitting with the bi-Langmuir equation was superior to the Langmuir equation for P_{NNB}-CB adsorption, indicating that there are two different adsorption sites on P_{NNB}-CB. The bi-Langmuir results show that the value of K_{d1} was 0.24 mg/mL, which only accounts for about one third of K_{d2}, indicating a stronger affinity between lipase and CB molecules than that between lipase and butyl groups. The results illustrate that the ligand CB was indispensable and was efficient for the affinity precipitation of lipase.

3.5. Optimization of desorption conditions

Desorption of bound lipase in different eluants (pH 8.0) is shown in Table 2. It can be seen that elution recoveries of lipase were not satisfactory with 1 mol/L NaCl and KSCN, which is consistent with the observation that addition of NaCl enhanced adsorption of lipase on P_{NNB}-CB. The optimal elution condition was 0.01 M phosphate buffer solution, pH 8.0, containing 20% ethylene glycol. 90.4% of the bound lipase was desorbed from P_{NNB}-CB and the specific activity of the eluted lipase reached 325.0 U/mg without obvious loss of activity. Contrary to the conditions promoting adsorption, a low salt concentration and 20.0% glycol would reduce the attraction between lipase and CB and result in higher elution recovery.

3.6. Reusability of P_{NNB}-CB

From an economic benefits point of view, the reusability of a polymer is the most important consideration for its application in an industrial process. Here, six cycles of

Table 1. Parameters of the equilibrium isotherms

Equation	q_1 (mg/g)	q_2 (mg/g)	K_{d1} (mg/mL)	K_{d2} (mg/mL)	q_0 (mg/g)	K_d (mg/mL)	Correlation coefficient
Langmuir(P_{NNB}-CB)	—	—	—	—	29.24	0.21	0.96
Langmuir(P_{NNB})	—	—	—	—	4.35	0.65	0.97
Bi–Langmuir(P_{NNB}-CB)	26.88	3.64	0.24	0.76	—	—	0.98

Table 2. Elution of bound lipase on CB-P_{NNB}

Elution agents	1.0 M NaCl	1.0 M KSCN	20% ethylene Glycol and 1 M NaCl	20% ethylene Glycol in 0.01 M phosphate buffer
Lipase recovery (%)	21.8	22.6	53.7	90.4
Specific activity (U/mg)	310	306	326	325

Table 3. Purification of lipase by affinity precipitation

	Initial lipase solution	Purified lipase	Purification efficiency
Specific activity	21 U/mg	336 U/mg	16 times
Total activity	402 U	328 U	81.6%

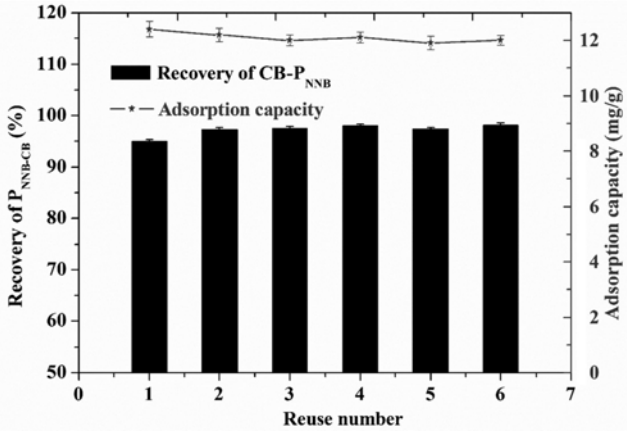

Fig. 5. Reusability of P_{NNB}-CB and stability of the adsorption capacity. Adsorption conditions: pH 5.0 0.02 mol/L phosphate buffer, ligand density 18.4 μmol/g polymers without NaCl, 20°C, 120 rpm shaking for 2 h. Desorption conditions: 0.01 mol/L phosphate buffer solution, pH 8.0, containing 20% ethylene glycol.

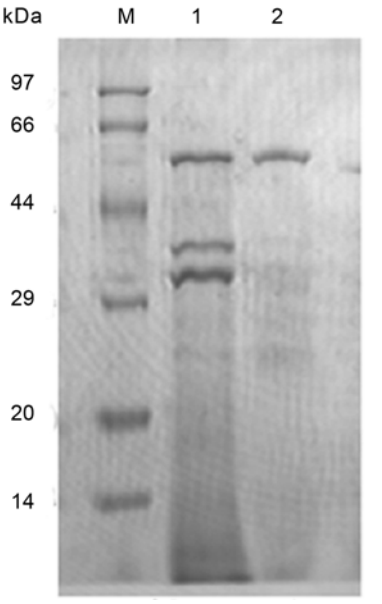

Fig. 6. SDS-PAGE analysis of purified lipase. Lane M: protein molecular marker; lane 1: crude lipase; lane 2: purified lipase.

adsorption-desorption were completed according to the above methods. As shown in Fig. 5, the adsorption of lipase on P_{NNB}-CB only slightly decreased from 12.4 mg/g in the first run to 12.0 mg/g in the sixth cycle. The adsorption capacity for lipase was quite stable over six cycles and the recoveries of P_{NNB}-CB were maintained in the range of 95 ~ 99%. Based on these results, it is concluded that P_{NNB}-CB is suitable for large-scale operations.

3.7. Application of P_{NNB}-CB to purification of lipase

According to the optimal conditions stated above, P_{NNB}-CB with a ligand density of 18.4 μmol/g was used to purify lipase from crude porcine pancreatic material in 0.02 mol/L phosphate buffer at pH 5.0, kept in a shaking water bath (120 rpm) at 20°C for 2 h. The absorbed lipase was eluted by a 0.01 mol/L phosphate buffer solution at pH 8.0 containing 20% ethylene glycol. As shown in Table 3, a 16-fold purification factor and 81.6% lipase activity yield were obtained. The specific activity of the purified lipase reached 336 U/mg. Fig. 6 shows an SDS-PAGE separation of the purified lipase, in which a single purified protein band can be observed in Lane 2. According to the above data, P_{NNB}-CB can be applied effectively for the purification of lipase, giving high selectivity. Conventionally, ammonium sulfate fractionation, precipitation by ethanol, and chromatography [5,6,9] are applied to purify lipase. Compared with these methods, affinity precipitation can obtain lipase at high purity from large-volume and dilute crude material solutions and the method requires fewer centrifugation steps. Affinity precipitation by P_{NNB}-CB has good potential for application and a high purity of lipase has been obtained with only a single step.

4. Conclusion

We have developed an affinity precipitation strategy to purify lipase using a modified thermo-responsive polymer conjugated with a CB ligand. Affinity purification of lipase is based on the formation of an affinity complex between the lipase and P_{NNB}-CB. The purified lipase can thus be separated from its crude material by dissolution of the complex and elution of the lipase. P_{NNB}-CB was precipitated, separated, and regenerated, and electrophoretically pure lipase could be obtained by affinity precipitation. The significantly improved recovery of the polymer obtained is of great importance with respect to materials recycle in large-scale industrial separations. The purification strategy is feasible and efficient for purification of proteins of interest.

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