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Synthesis of thermo-sensitive copolymer with affinity butyl ligand and its application in lipase purification

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ABSTRACT

In this study, thermo-sensitive N-alkyl substituted polyacrylamide polymer P_{NNB} was synthesized by using N-hydroxymethyl acrylamide(NHAM), N-isopropyl acrylamide (NIPA) and butyl acrylate (BA) as monomers, and its low critical solution temperature (LCST) was controlled to be 28 °C. The recovery of the thermo-sensitive polymer was over 98%. Butanol as a hydrophobic ligand was covalently attached onto polymer P_{NNB} and butyl ligand density was 80 μmol g^{-1} polymer. The affinity polymer was used for purification of lipase from crude material. Optimized condition was pH 7.0, 35 °C adsorption temperature, 120 min adsorption time and 0.5 mg ml^{-1} initial concentration of lipase. The adsorption isotherm accords with a typical Langmuir isotherm. The maximum adsorption capacity (Q_m) of the affinity polymer for lipase was 24.8 mg g^{-1} polymer. The affinity copolymer could be recycled by temperature-inducing precipitation and there was only about 6% loss of adsorption capacity after five recyclings. Specific activity of lipase was improved from 14 IU mg^{-1} to 506 IU mg^{-1} protein, and its recovery achieved 82%. The affinity polymer is suitable for the purification of target proteins from the crude material with large volume and dilute solution.

1. Introduction

Developing new methods for protein purification in large scale is still a challenge. The affinity precipitation could significantly reduce volume of initial material and improve the purification efficiency [1]. The technique is known for over 20 years, and has recently received more attention with the development of new affinity polymer materials [2].

Affinity precipitation is achieved by a reversibly soluble polymer with immobilized ligands, and reversibly soluble polymer means that solubility and precipitation of the polymer can be shifted by changing environment conditions such as pH, temperature, ionic strength or organic solvent etc. Among these reversibly soluble-insoluble polymers, temperature-responsive polymers have been extensively studied [3], and its reversible state of solubility and precipitation is achieved by controlling temperature. The phenomenon is not yet completely understood. However, stability of H-bridges between a polymer and water molecules weaken with increasing temperature, which contribute to the observed phenomenon [4]. Chen and Hoffman [5] synthesized a copolymer of NIPAM and N-acryloxysuccinimid. Protein A immobilized on this copolymer enabled the capture IgG by thermo-precipitation of affinity complex. Vaidya et al. prepared thermo-sensitive polymer containing acetamido group and applied it to lysozyme purification by affinity thermo-precipitation [6]. Shen and Cao synthesized a new thermo-sensitive polyacrylamide derivative for affinity precipitation and applied it to purification of lysozyme [7]. The group also prepared a light-sensitive and reversibly soluble copolymer and applied it to purification of lysozyme from egg-white [8].

Lipase is an important enzyme in biological systems, where it catalyses hydrolysis of triacylglycerol to glycerol and fatty acids. The enzyme is distributed in higher animals, plants and microorganism in which it plays a key role in lipids metabolism. The enzyme has been widely used for biotechnology industry [9,10]. Lipase is a higher hydrophobic protein, compared with conventional proteins and has an inherent affinity toward hydrophobic ligand. Separation of lipase from an aqueous medium is based on the association between hydrophobic groups on the surface of proteins and hydrophobic groups on the affinity media. The type of hydrophobic group incorporated into a support is an important variable that influences adsorption of protein on a support. In hydrophobic interaction, commonly used ligands are mainly butyl, octyl and phenyl groups.

In this study, a new thermo-sensitive copolymer (P_{NNB}) was developed, which consisted of N-hydroxymethyl acrylamide (NHAM), N-isopropyl acrylamide (NIPA) and butyl acrylate (BA) monomers. And butyl group as hydrophobic ligands was attached

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on P_{NNB} to obtain an affinity polymer. Lipase was purified with the thermo-sensitive affinity copolymer and some interesting results have been obtained.

2. Materials and methods

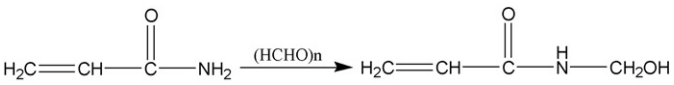
2.1. Materials

N-isopropyl acrylamide (NIPA) and N-hydroxymethyl acrylamide (NHAM) were synthesized by ourselves. Acrylate (BA) was from Shanghai Ling Feng Chemical Reagent Co. Ltd. (Shanghai, China). Pure porcine pancreas lipase was obtained from Kaiyang Biotechnology Co. Ltd. (Shanghai, China), and crude material of porcine pancreas lipase (14 U mg^{-1}) was purchased from Dongwu Biochemical Products Company (Suzhou, China). And all other reagents used were reagent grade.

2.2. Methods

2.2.1. Synthesis of N-hydroxymethyl acrylamide (NHMA)

The mixture of 1.0 ml xylene and a little sodium was heated to melting state, and then, 30.0 ml methylene chloride, 3.55 g acrylamide and 1.5 g polyformaldehyde were added into it. The reaction was carried out for 30 min. After the reaction finished, the solution was filtrated. The filtrate was placed at 4 °C to precipitate out product, and then the product was filtrated and dried [11]. The reaction formula is as follows:



2.2.2. Synthesis of copolymer P_{NNB}

Mixture containing specified amount of three monomers (NIPA, NHMA and BA), AIBN as polymerization initiator and ethanol as solvent were poured into a flask with a nitrogen atmosphere maintained for 10 min. The reaction was carried out for 24 h at 60 °C and then ethanol was removed by distillation. The residue was dissolved in acetone and precipitated in excessive hexane. Then, the precipitates were collected and dried.

2.2.3. Immobilization of ligand on polymer P_{NNB}

Various amounts of Butyl glycidyl ether were dissolved in acetone in the range of 0.024–0.480 mg ml^{-1}. 0.1 g ml^{-1} polymer and 0.2 ml ml^{-1} boron trifluoride was added into the solution. The reaction was carried out for 45 min at 45 °C. After the reaction was finished, the mixture was poured into excessive hexane to precipitate the polymer with immobilized ligands from the reaction solution. Residual butyl glycidyl ether was determined by the classical hydrochloric acid–acetone method [12] and butyl ligand density was calculated according to mass balance.

2.2.4. Adsorption–desorption of lipase on affinity polymer

2.2.4.1. Adsorption of lipase on polymer P_{NNB}. The enzyme concentration was 0.5 mg ml^{-1}. Adsorption experiments were carried out for 2 h with continuous stirring. Adsorption of lipase on the polymer was investigated at pH 4–8 in 20 ml acetate (0.1 M, pH 4.0–5.0) or 20 ml phosphate buffers (0.1 M, pH 6.0–8.0). The effect of temperature on lipase adsorption was investigated in 20 ml phosphate buffer (0.1 M, pH 7.0) at 20–50 °C. The amount of adsorbed lipase on the polymer was determined by measuring absorbance at 280 nm at the initial and final concentrations of free protein in the supernatant.

2.2.4.2. Recycling of copolymer P_{NNB} and desorption of lipase. The recovery of polymer P_{NNB} was determined by constant dry weight of the polymer after thermo-precipitation. To determine reusability of the polymer, the adsorption and desorption cycles was repeated five times using the same polymer. The lipase desorption experiments were carried out by using a mixture containing 50% of ethylene glycol containing 1.0 M NaCl. The solution of desorption was stirred magnetically at 100 rpm at 25 °C for 120 min, and then was heated to 32 °C to precipitate the polymer and the lipase concentration in eluate was determined. The desorption recovery of lipase was calculated according to the amount of lipase released divided by the amount of lipase adsorbed on the polymer.

2.2.5. Activity assays of lipase

Activity of lipase was determined by olive oil hydrolysis. Olive oil emulsion (5%, w/v) was prepared by mixing olive oil with a polyvinyl alcohol 4% (w/v, dissolved in distilled water). The assay mixture consists of 20 ml emulsion and 2.0 ml free enzyme or 2.0 ml adsorbed enzyme. The mixture was stirred for 30 min at 37 °C. The fatty acids produced were determined by titration with 0.05 M NaOH solution. One lipase unit was expressed as releasing 1 μmol fatty acid per minute in the assay conditions, and the specific activity was expressed as lipase units per mg protein.

3. Results and discussion

3.1. Synthesis of polymer P_{NNB}

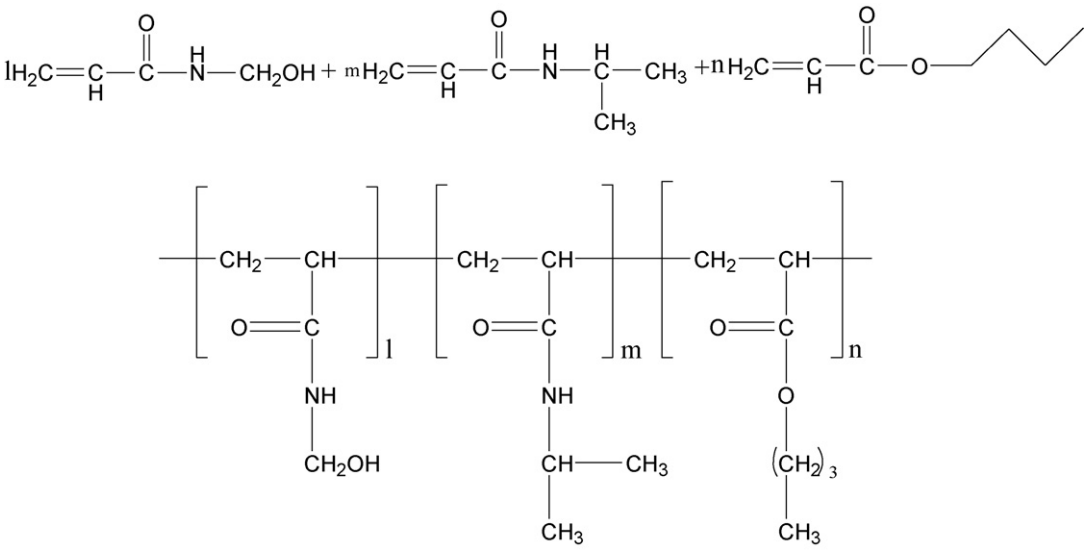
Among the three blocks, NIPA provides thermo-sensitive character of copolymer P_{NNB} and NHAM brings hydroxyl groups which could be used to immobilize hydrophobic ligand, while BA is used to control lower critical solution temperature (LCAT). Table 1 shows characteristic of the polymers with different monomers ratio. All of polymers with different monomers ratio are reversibly soluble. LCST decreased with the increase of NHMA due to the ratio change of hydrophilic monomer and hydrophobic monomer. The increase of BA also caused the same phenomenon. In the subsequent experiments, we chose #1 polymer because of its high recovery and good biocompatibility. The molecular weight of the polymer measured by viscosity method was 3.0×10^4 Da. The possible reaction formula is as

Table 1
Characteristic of polymers with different monomer ratio.

Number	Monomer			LCST (°C)	Recovery
	NHMA (mmol)	NIPA (mmol)	BA (mmol)		
#1	1.98	70	15.6	28.6	98.6%
#2	1.98	70	7.8	36.4	96.8%
#3	0.99	70	15.6	34.2	96.2%

Note: LCST was low critical solution temperature of polymers. Recovery of the polymer was determined by constant dry weight of the polymer after thermo-precipitation.

follows:



3.2. Characterization of copolymer P_{NNB}

The FTIR spectra of copolymer P_{NNB} with butyl ligand are presented in Fig. 1. FTIR spectra of the copolymer have the characteristic absorption around 3391 cm^{-1} and 1700 cm^{-1}, which indicates that the copolymer contains hydroxyl groups and acylamide groups. The FTIR spectrum of the copolymer has characteristic methyl group and methylene group absorption bands at 2960 cm^{-1}, 2870 cm^{-1} and 2930 cm^{-1}, respectively. It could be concluded that the expected copolymer P_{NNB} has been synthesized successfully.

3.3. Butyl ligands immobilized on polymer P_{NNB}

Here we used a novel method to introduce a hydrophobic group onto the polymer. The merit of this method is to avoid activation steps during immobilizing indirectly butyl ligand on polymer

Table 2
Influence of initial ligand concentration on linked ligand density of polymer.

Initial concentration of butyl glycidyl ether (mg ml^{-1})	Ligand density (μmol g^{-1}) polymer
0.024	19.8
0.048	43.4
0.096	79.6
0.192	174.5
0.384	314.2
0.480	533.6

Note: Different concentrations of butyl glycidyl ether were dissolved in acetone solution, and 0.1 g ml^{-1} polymer and 0.2 ml ml^{-1} boron trifluoride was added into the solution. The immobilization reaction was carried out for 45 min at 45 °C. The density of immobilized ligand was determined by the classical hydrochloric acid–acetone method.

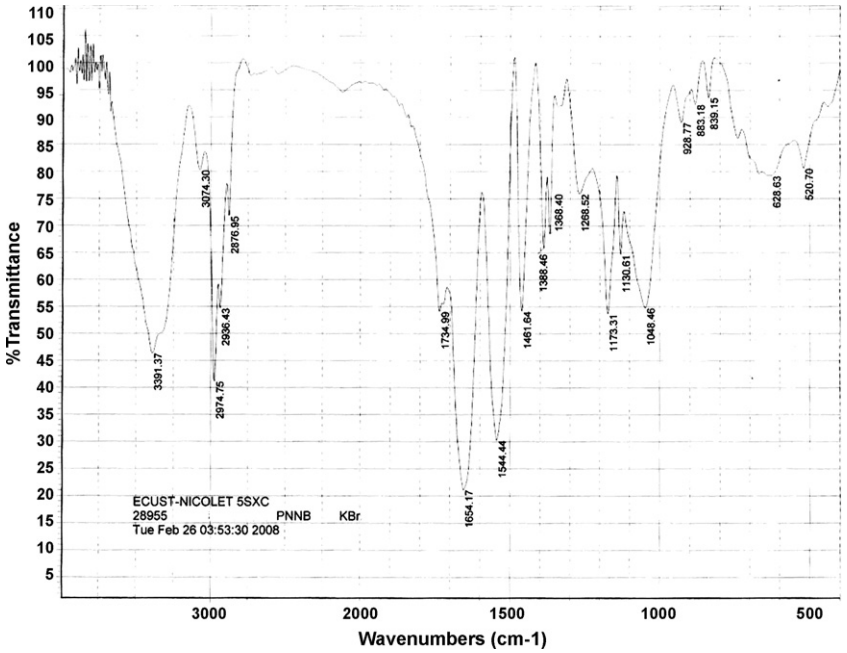


Fig. 1. FTIR spectra of copolymer with butyl ligand. FT-IR spectrometer was Magna-IR 550, Nicolet and KBr tabletting method was used.

Table 3
Effect of ligand density on lipase adsorption.

Number	ligand density (μmol g^{-1})	adsorption capacity (mg g^{-1})
1	19.8	14.3
2	43.4	19.6
3	79.6	23.8

Note: Polymer concentration was 20 mg ml^{-1}, initial concentration of lipase was 0.5 mg ml^{-1}, adsorption temperature was 35 °C, and adsorption time was 120 min.

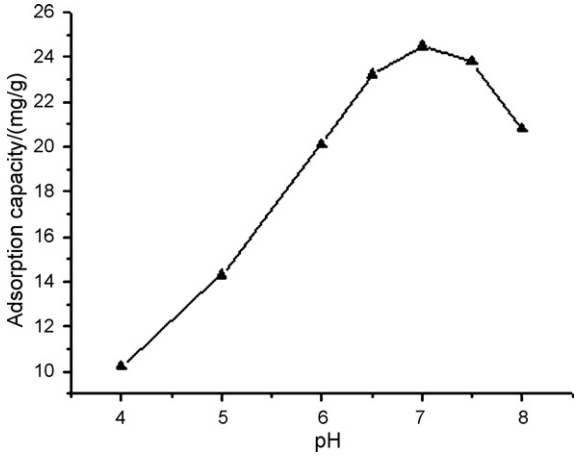


Fig. 2. Effect of pH on the adsorption capacity of polymer P_{NNB}. Polymer concentration was 20 mg ml^{-1} with 80 μmol g^{-1} ligand density, initial concentration of lipase was 0.5 mg ml^{-1}, adsorption temperature was 35 °C, and adsorption time was 120 min.

P_{NNB}. Butyl ligands and the copolymer were connected by epoxy group, which acts as a spacer between the polymer and the ligand, and this spacer could increase contact between the ligands on polymer and lipase. Butyl group is a kind of hydrophobic ligand, and it could be covalently immobilized on the polymer. Different ligand density could be obtained by changing initial concentration of butyl glycidyl ether (Table 2). When initial concentration of butyl glycidyl ether changed from 0.024 mg ml^{-1} to 0.480 mg ml^{-1}, the ligand densities immobilized onto the copolymer from 19.8 μmol g^{-1} polymer to 533.6 μmol g^{-1} polymer (Table 2). The reaction formula is as follows:

3.4. Adsorption of lipase onto polymer P_{NNB}

3.4.1. Effect of ligand density on adsorption of lipase

The effect of ligand density on adsorption of lipase is shown in Table 3. High adsorption capacities were obtained at high ligand densities on the polymer. Adsorption capacity of lipase increases with ligand density of polymer below 100 μmol g^{-1} polymer because high ligand density polymer could provide more hydrophobic affinity sites than the lower one. However, solubility of the polymer decreased very quickly with higher ligand densities because butyl ligand is a hydrophobic group. So, the ligand densities of 80 μmol g^{-1} polymer was used in following experiments.

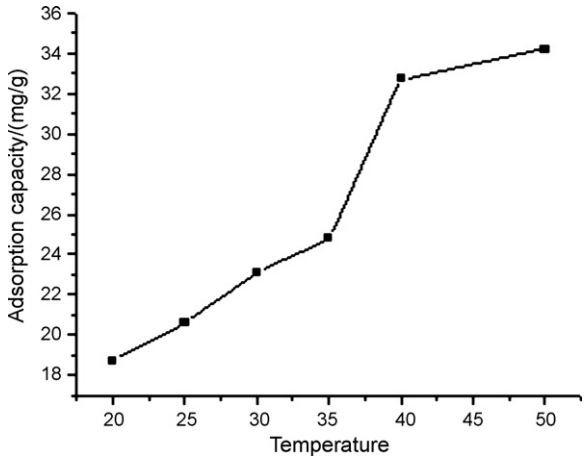


Fig. 3. Effect of temperature on the adsorption capacity of polymer P_{NNB}. Polymer concentration was 20 mg ml^{-1} with 80 μmol g^{-1} ligand density, initial concentration of lipase was 0.5 mg ml^{-1}, adsorption time was 120 min, and pH was 7.0.

3.4.2. Effect of pH and temperature on adsorption of lipase

Fig. 2 shows the amount of lipase adsorbed onto the polymer at different pH values. The maximum lipase adsorption was obtained at pH 7.0, almost equal to the isoelectric point of the lipase (6.9), so electrostatic interactions between lipase and the polymer could be minimized, and the affinity force is only attributed to hydrophobic affinity. Lower lipase adsorption ability was observed for the polymer in all other tested pH.

The effect of temperature in hydrophobic interaction is also important. At higher temperatures, the proteins expose hydrophobic amino acid residues on its surface, thus, the contact area between the protein and affinity ligand on the polymer may increase, resulting in higher affinity possibility. Lipase shows a greater adsorption capacity at higher temperature (Fig. 3). From 20 °C to 50 °C, adsorption capacity of the affinity polymer for lipase increased sharply, reached 34.2 mg g^{-1} polymer. The increase of adsorption capacity for lipase at higher temperature indicates that binding between the protein and butyl ligand is mainly hydrophobic interaction.

3.5. Effect of pH and temperature on catalytic activity of lipase

The effect of pH on activity of free and adsorbed lipase was determined in the range of pH 4.0–9.0. The result is presented in Fig. 4. The maximum activity was observed at pH 7.5 for both free and adsorbed lipase. The effect of temperature on activity of lipase was investigated in the range of temperature 20–50 °C. The optimum temperature for free enzyme was about 35 °C, while for the adsorbed enzyme was about 40 °C (Fig. 5). This observation could be explained by the creation of conformational limitations on the lipase as a result of hydrophobic interactions between the enzyme and the polymer.

3.6. Reusability of the polymer

In order to investigate reusability of the polymer, the adsorption–desorption cycles of lipase was repeated five times. The lipase adsorbed onto the polymer was eluted by 50% ethylene glycol containing 1.0 M NaCl. Fig. 6 showed that the affinity polymer was

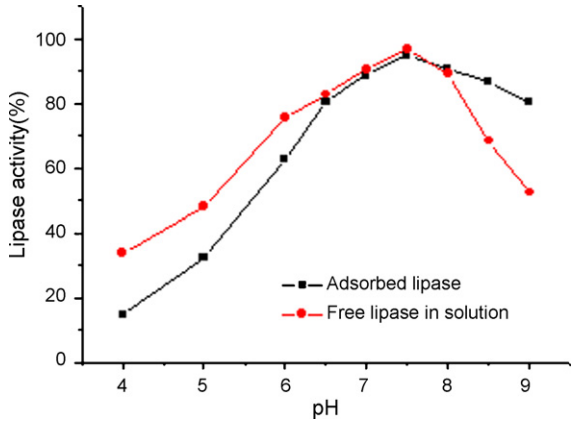


Fig. 4. Influence of pH on activities of free and adsorbed lipase. Free lipase was 0.5 mg ml^{-1}, polymer with 80 μmol g^{-1} ligand density adsorbed 25 mg lipase per gram and it concentration was 20 mg ml^{-1}, thus adsorbed lipase was also 0.5 mg ml^{-1}. Temperature was 35 °C.

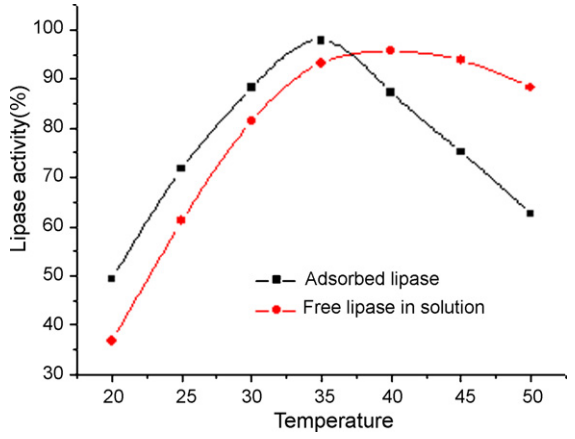


Fig. 5. Effect of temperature on activities of free and adsorbed lipase. Free lipase was 0.5 mg ml^{-1}, polymer with 80 μmol g^{-1} ligand density adsorbed 25 mg lipase per gram and it concentration was 20 mg ml^{-1}, thus adsorbed lipase was also 0.5 mg ml^{-1}. pH was 7.5.

repeatedly used in lipase purification. The elution recovery could reach 94.5%, and adsorption capacity of the polymer decreased only 6% related to initial adsorption capacities after the five repeated adsorption–desorption runs.

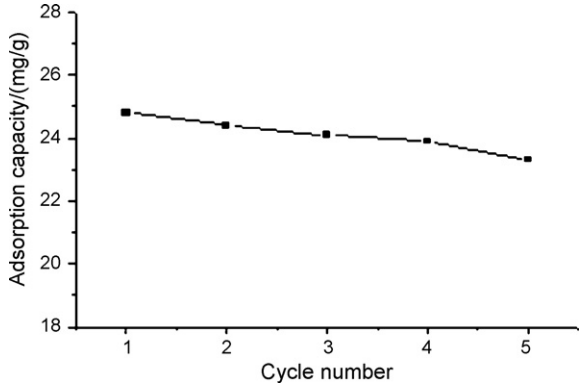


Fig. 6. Reuse of affinity polymer for lipase adsorption. The adsorption and desorption of lipase was repeated five times using same polymer, polymer concentration was 20 mg ml^{-1} with 80 μmol g^{-1} ligand density, concentration of lipase in adsorption was 0.5 mg ml^{-1}, adsorption time was 120 min, and pH was 7.0.

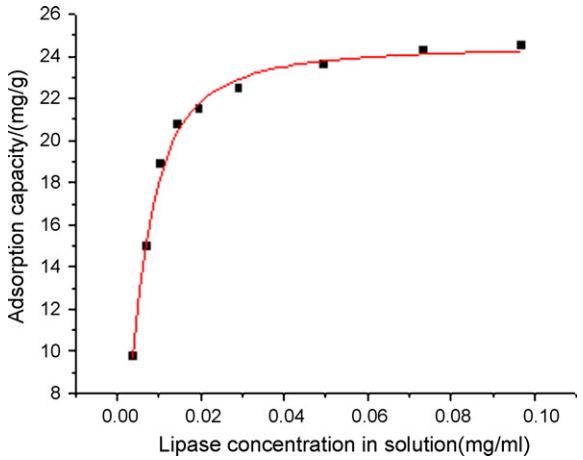


Fig. 7. Affinity adsorption isotherm of polymer P_{NNB}. Polymer concentration was 20 mg ml^{-1} with 80 μmol g^{-1} ligand density, adsorption time was 120 min, and pH was 7.0.

3.7. *Affinity adsorption isotherm*

Fig. 7 shows adsorption isotherm of lipase binding on affinity polymer P_{NNB}. The curve obeys Langmuir isotherm. A Langmuir equation could be applied to simulate experimental data as following:

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

where q is adsorption capacity of affinity polymer P_{NNB}, C is concentration of unbound lipase in solution, Q_m is maximum capacity of the affinity polymer and K_d is dissociation constant.

Simulating Eq. (1) with experiment data, we obtained Q_m to be 24.8 mg g^{-1} polymer and K_d to be 0.00503 mg ml^{-1}. The molecular weight of lipase was about 50,000 Da. So, Q_m was equivalent to 0.496 μmol g^{-1} polymer and K_d was equivalent to 0.1 μmol L^{-1}, and almost 160 butyl groups bind one lipase molecule by hydrophobic interaction, which indicates multivalent binding behavior. It may also be caused by space hindrance of the large size and high molecular weight of lipase.

3.8. *Purification of lipase from its crude material*

The affinity polymer P_{NNB} was applied to purification of lipase from its crude material. Table 4 shows that specific activity of lipase increased from 14.0 U mg^{-1} protein in crude material to 508.2 U mg^{-1} protein, and its purification factor reached 36.3. When initial concentration of crude lipase was 0.5 mg ml^{-1}, yield could reach 82.6%. Higher recovery could be obtained by adding more copolymer into the solution or decreasing the initial lipase concentration. Fig. 8 shows the purification effect of lipase with SDS-PAGE electrophoresis. The molecular weight of lipase was around 50 kDa. This indicates that the affinity precipitation was a feasible method in purification of lipase. We can obtain electrophoresis pure lipase by only one step purification.

Table 4
Purification effect of lipase from its crude material by affinity precipitation.

	Specific activity (IU/mg)	Purification factor	Yield (%)
Crude enzyme	14.0	–	
After purification	508.2	36.3	82.2

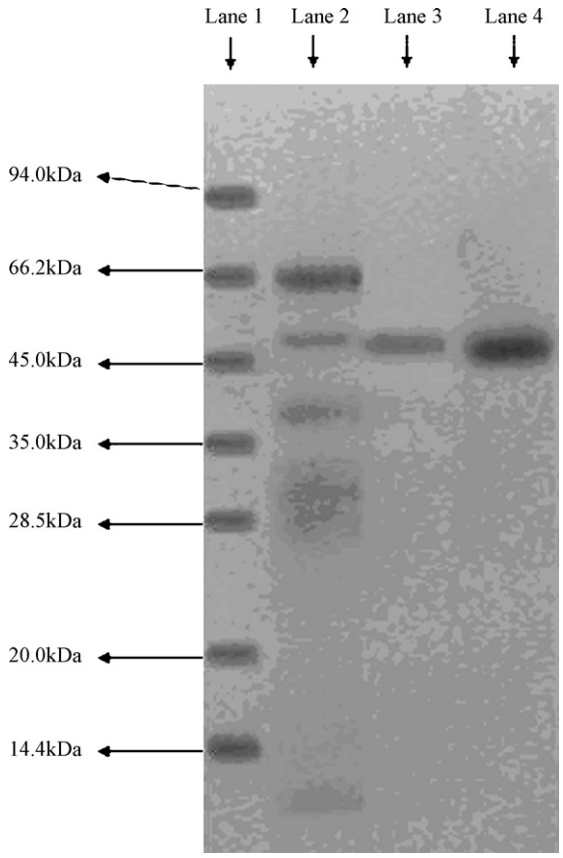


Fig. 8. Purification effect of lipase with affinity precipitation. Lane 1: Protein mark, Lane 2: crude enzyme, Lane 3: lipase after purification, Lane 4: pure enzyme.

4. Conclusions

Thermo sensitive copolymer P_{NNB} was synthesized with recovery 98.6% by controlling monomer ratio of NHMA/NIPA/BA (2:70:15). The LCST of the polymer was about 28.6 °C, and its molecular weight was about 3.0×10^4 Da. The hydrophobic butyl group as ligand was immobilized onto the polymer with the ligand density 80 μmol g^{-1} polymer. At pH 7.0, 35 °C and 0.5 mg ml^{-1} of initial lipase concentration, the adsorption capacity of lipase reached 24.5 mg g^{-1} polymer. Adsorbed lipase was eluted by using 50% ethylene glycol containing 1.0 M NaCl, and elution recovery could reach 94.5%. Finally, we applied this polymer to the purification of lipase, SDS-PAGE electrophoresis showed good purification results. This copolymer could be recycled by temperature-inducing and repeatedly used in lipase purification.

Acknowledgements

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