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One-step purification of YLLIP2 isoforms from *Candida* sp. 99–125 by polyethyleneimine modified poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) monolith

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ABSTRACT

The extracellular lipase *Yarrowia lipolytica* (YLLIP2) crude extract was efficiently separated and purified from *Candida* sp. 99–125 by one-step ion-exchange chromatography on polyethyleneimine (PEI) functionalized monolithic columns. The preparative conditions for the functionalization of monoliths were optimized, including PEI molecular mass, PEI concentration, modification time and temperature. The monolithic skeleton was prepared *in situ* by polymerization of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EGDMA) with a volume ratio of 8:2. Heptane was used as the porogen. PEI 30 kDa with the concentration of 10% (v/v) was applied for the modification of the monolith at 55 °C for 12 h. Lipase (EC.3.1.1.3) from *Candida* sp. 99–125 was separated to four isoforms (isoform A, isoform B, isoform C and isoform D). As analyzed on non-denaturing PAGE and MALDI-TOF–MS, the four isoforms are homogenous and have the same molecular mass of approximate 38 kDa. The monoliths can afford direct crude lipase loading without increasing too much back pressure, which explores the great potential of the application of monoliths for one-single step fast separation and purification of complicated proteins.

1. Introduction

Lipases (EC 3.1.1.3), which hydrolyze triacylglycerol at water/oil interfaces to yield glycerol and long chain fatty acids, are extensively employed for the hydrolysis [1], alcoholysis [2,3], esterification [4], transesterification [5] of carboxylic esters as well as resolution of racemic mixtures [6]. Following proteases and carbohydrases, lipases are considered to be the third largest group of enzymes based on total sales volume [7]. Lipases secretion in *Yarrowia lipolytica* was first reported in 1948 by Peters and Nelson [8,9], who described a single glucose-repressible lipase activity with an optimum pH value around 6.2–6.5.

In most commercial applications, lipases are immobilized on different carriers or lyophilized as crude products, containing different amounts of impurities. Therefore it is desirable to purify lipase for biochemical characterization of wild-type and mutant enzymes [10]. Ion-exchange chromatography [10–18], hydrophobic chromatography [17], size exclusion chromatography [11,19] as well as affinity chromatography [20,21] were demonstrated to be efficient methods for the separation and purification of lipases. In most cases, lipase purification procedures include several chromatography steps [11–13,15–18]. Aloulou et al. [12] used two steps of separation, gel filtration and cation exchange chromatography, to purify *Y. lipolytica* (YLLIP2) and obtained four isoforms. Fu et al. [18] also adopted two steps of ion-exchange chromatography for the separation and purification of lipase from *Candida* sp. 99–125 and got four isoforms. Only a few methods [10,14,21,22] were reported for the purification of lipases in a single step, which was always for enzymes produced at high levels in the culture medium of genetically modified microorganisms or cells.

Monolithic stationary phases, directly cast in tubes by radical polymerization have high permeability and good mass transfer, leading to improved resolution at high linear flow-rates. With the functional epoxy groups, monoliths based on poly(glydiyl methacrylate-co-ethylene dimethacrylate) can be modified to ion-exchangers [23,24], reversed-phase chromatography media [25,26] and affinity chromatography media [27]. One of the limitations for organic polymer monolith is its low binding capacities for proteins [28]. The alternative way to increase the binding capacity may be achieved by the immobilization of a polymer chain to the reactive sites on the surface of the monolith, which results in larger surface ligand density.

In this paper, direct immobilization of polymer chains on the monolith was performed to improve the binding capacity for lipases. The monolithic column was functionalized by

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polyethyleneimine (PEI) for one-step separation and purification of crude lipase. The dynamic binding capacity and adsorption property were characterized. Influences of PEI molecular mass and PEI concentration on the separation of lipase were also investigated. Four pure isoforms can be obtained from *Candida* sp. 99–125 in one-single step. The monoliths can afford direct crude lipase loading without increasing too much backpressure thanks to good mass transfer. After flushing with high concentration of salt solution, the monoliths can be regenerated. Compared to normal performance ion-exchange columns, the PEI modified monolith is applicable in high performance chromatography with better separation resolution and efficiency. Compared to previous works [12,18,29], the crude lipase was separated into four isoforms in one-single step instead of two or more chromatography steps.

2. Materials and methods

2.1. Materials

Candida sp. 99–125 (CGMCC 1470) was obtained from our laboratory [29–33] and registered at the China General Microbiological Culture Collection Centre (CGMCC). Glycidyl methacrylate (GMA, 99%) and ethylene glycol dimethacrylate (EGDMA, 99%) were purchased from Acros (Morris Plains, NJ, USA). 2,2-Azobis-(2-methylpropionitrile) (AIBN) was purchased from Beijing Chemical Reagent Plant (Beijing, China) and re-crystallized with ethanol before using. Polyethyleneimines (PEIs) with different molecular mass (0.6, 2, 20, 30 and 100 kDa) were provided by Acros (Morris Plains, NJ, USA). Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Molecular mass markers for electrophoresis were from Bio-Rad. All other reagents used in experiments were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China.

2.2. Preparation and modification of monolithic columns

The preparation and modification of monolithic columns were performed according to our previous work [34] with some modifications. The monolithic skeleton was prepared *in situ* by polymerization of functional monomers and cross-linkers, GMA and EGDMA with a volume ratio of 8:2, in a 100 mm × 4.6 mm i.d. stainless-steel chromatographic column tube. Heptane was used as the porogen. Functional monomers and porogenic solvent were mixed in the volume ratio of 4:6 to prepare a uniform solution in which AIBN (1% (w/v), with respect to monomers) was dissolved. The column was sealed with a polystyrene cap in one end, filled with 1.7 mL of the monomer solution and degassed by ultrasonication for 15 min followed by sealing the other end of the tube with another cap. The polymerization was allowed to proceed at 60 °C for 12 h followed by replacing the polystyrene caps with column end fittings. The column was connected to a HPLC system. The porogenic solvent and other soluble compounds present in the formed monolith were removed by pumping tetrahydrofuran (50 mL) through the column. Subsequently, the column was washed with water (20 mL) and filled with different concentrations of PEIs solutions. The modification was performed in a water bath at different temperatures for different times followed by washing with water and 0.01 M Tris–HCl buffer (pH 7.6).

2.3. Determination of enzyme activity

Lipase activity was measured by titrimetric assay using an olive oil emulsion [35]. Olive oil [25%, v/v] was emulsified in distilled water containing 2% (w/v) of poly (vinyl alcohol) (PVA) in a homogenizer for 6 min at maximum speed. Then the enzyme solution (1 mL), pure or diluted, depending on the quantity of lipase, was added to 5 mL of substrate emulsion and 4 mL of 100 mM phosphate buffer, pH 8.0 (K_2HPO_4–KH_2PO_4). Samples were incubated for 10 min at 40 °C. The reaction was stopped by adding 15 mL ethanol. Enzyme activity was determined by titration of the fatty acid released with 50 mM NaOH. One activity unit of lipase was defined as the amount of enzyme, which released 1 mmol of fatty acid per minute under assay conditions [29].

2.4. Purification of the lipase

2.4.1. Preparation of the lipase crude extract

Lipase culture was obtained from our lab [29–33]. About 300 mL of *Y. lipolytica* culture broth was centrifuged at 4500 × *g* for 30 min at 4 °C to discard the cells. The supernatant was constantly stirred at 0 °C and treated with three volumes of ice-cold acetone. The precipitate which contained the enzyme activity was collected by centrifugation at 12,000 × *g* for 30 min at 4 °C, washed twice by ice-cold acetone to extract the oil and dried at 4 °C to obtain the crude enzyme. About 10 g of crude powder was dissolved in 40 mL of 10 mM Tris–HCl buffer (pH 7.6). After centrifugation (12,000 rpm min^{-1}, 20 min) of the samples, the supernatant was collected and used as lipase crude extract.

2.4.2. Fast separation of lipase by PEI modified ion-exchange chromatography

All chromatographic steps were run on ÄKTA basic 100 (Amersham biosciences) with a UV detection at 280 nm. The clear sample (15 mL) obtained in the previous step was loaded on PEI modified ion-exchange monolithic column (100 mm × 4.6 mm i.d.) equilibrated with 10 mM Tris–HCl buffer (pH 7.6) at the flow rate 1 mL min^{-1}. The unbound protein was washed out with 25 mL of the equilibration buffer. Subsequently a linear gradient of 0–1.0 M NaCl in the same buffer was used to elute bound lipase activity and fractions of 6 mL were collected. After the gradient the proteins strongly bound to the column were washed out with equilibration buffer containing 2 M NaCl.

2.5. Measurement of dynamic binding capacity

To determine the dynamic binding capacity of PEI functionalized monolith, frontal analysis of the column was carried out with 2 mg mL^{-1} BSA in pH 7.6 Tris–HCl buffer. The binding capacity (Q) was calculated by Eq. (1):

$$Q = \frac{(V_{HB} - V_0)c}{m} \quad (1)$$

where V_{HB} (mL) is the half breakthrough volume of BSA, V_0 the dead volume the column, c (mg mL^{-1}) the BSA concentration in the mobile phase and m (g) is the dry weight of the monolithic media.

2.6. Gel electrophoresis

Non-denaturing electrophoresis was performed with a 12% polyacrylamide gel on a vertical mini gel apparatus (Bio-RAD) at 150 V for 1 h. Isoelectric focusing (IEF) was performed at 4 °C using a PROTEAN IEF cell (Bio-Rad, USA) using Readystrip IPG strips (Bio-Rad) with a pH range of 3–10. The gels were stained for protein detection with Coomassie Blue R-250 following standard procedures.

2.7. MALDI-TOF mass spectrometry

The mass spectra were recorded by using a time-of-flight delayed extraction MALDI mass spectrometer (Bruker Autoflex). The samples were treated according to Ref. [18].

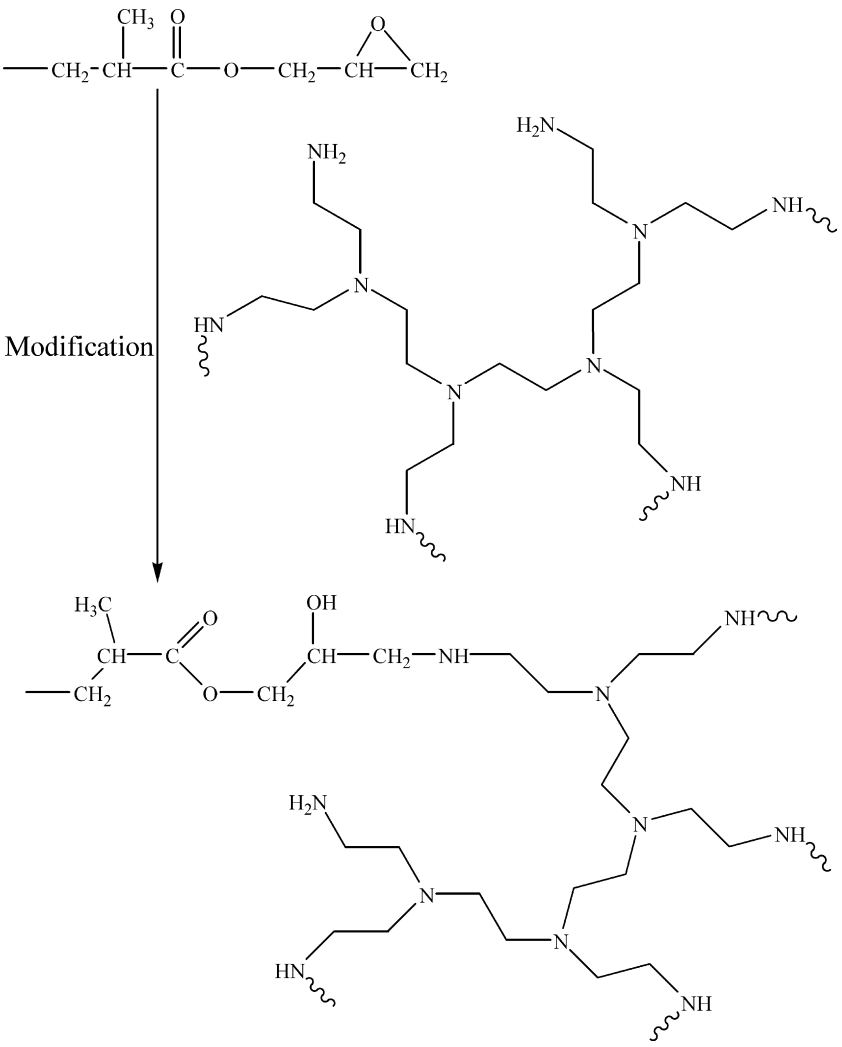

Fig. 1. The sketch of the modification reaction between poly(GMA-co-EGDMA) monolithic skeleton and PEI.

2.8. Protein assay

Protein concentration was determined by the Bradford method using bovine serum albumin as standard [36].

3. Results and discussions

3.1. Optimization of preparative conditions

During the functionalization process, the amine group of PEIs can react with the epoxy groups on the surface of the monolith to form polymer brush (Fig. 1). Varying the molecular mass, concentration of PEI as well as modification time and temperature can influence the length and amount of the polymer brush on the monolithic surface, and consequently affect the binding capacity of the column.

3.1.1. Effects of PEI molecular mass

To investigate influences of PEI molecular mass on the chromatographic performances of the monolith, PEIs with different molecular mass from 0.6 kDa to 100 kDa at the same concentration of 5% (v/v) were used for the modification of the monoliths. Dynamic binding capacities of BSA versus molecular mass of PEIs were calculated and depicted in Table 1. It is demonstrated that with the increase of PEI molecular mass, the binding capacities of the monoliths for BSA increase. Larger PEI molecular mass correspond to longer and more flexible polymer chain, displaying a larger surface area and a higher binding capacity for proteins. However, too long polymer chain (corresponding to a mass of 100 kDa) is not conducive to the modification of the monolith.

The monolithic columns modified by PEIs with different molecular mass were used for one-step separations of the lipase. The lipase activity was bound to the monolith resin. After eluting an unbound fraction without activity, the lipase activity was eluted with gradient elution from 0 to 1.0 M NaCl concentration. It is indicated that the ion-exchange monolith can fulfill the aim of one-single step of separation and purification of lipase into four isoforms, named isoform A, isoform B, isoform C and isoform D. This has obvious advantages over traditional multi-steps separation methods. PEI with the molecular mass approximate 30 kDa has the best separation efficiency for lipase. Too short or too long PEI polymer chain

Table 1
Dynamic binding capacities of BSA versus molecular mass of PEIs.

Molecular mass of PEIs (kDa)	BSA binding capacity (mg g^{-1})
0.600	17.4
2.00	32.2
20.0	38.6
30.0	41.5
100	35.8

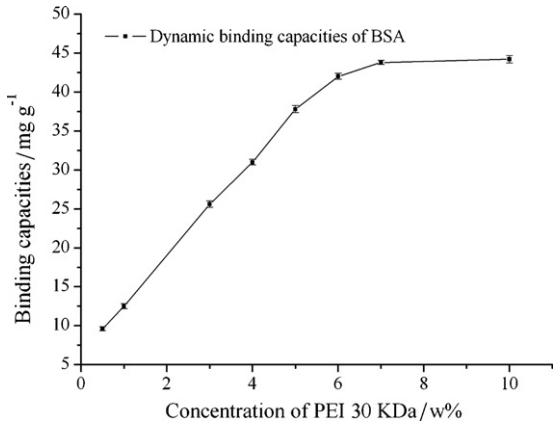

Fig. 2. Dynamic binding capacities of PEI functionalized monoliths for BSA versus concentrations of PEI 30 kDa.

is not favorable to the lipase separation. In comparison with PEI 30 kDa, PEI 20 kDa has no high selectivity for the four isoforms. It might arise from the fact that the chain length of PEI 20 kDa is not quite suitable for the separation of the four isoforms.

3.1.2. Effects of PEI concentration

To study the influences of PEI concentration on the property of modified monolith, PEI 30 kDa with concentrations ranging from 0.5% to 10% (wt%) were used as the modifiers. Fig. 2 depicts the binding capacities of monoliths for BSA with changes of PEI concentration. With the increase of PEI concentration, the binding capacity of the monolith for BSA increases. Higher PEI concentration corresponds to higher PEI ligand density, resulting in larger binding capacity. However, the PEI solution with a concentration higher than 10% (v/v) has high viscosity and cannot be used to achieve the modification of the monolith. Too high back pressure might destroy the skeleton structure of the monolithic resin. Therefore, 10% (v/v) PEI 30 kDa is chosen for the modification of the monolith in our further study.

3.1.3. Effects of modifying time

The modifying time directly influenced the ligand density of the monolith. In this section, the property of the monolith was studied with changes of the modifying time. PEI 30 kDa with a concentration of 10% (v/v) was used for the modification of the monolith. As shown in Fig. 3, from 0 h to 12 h, the binding capacity of BSA increases with the increase of modifying time. During

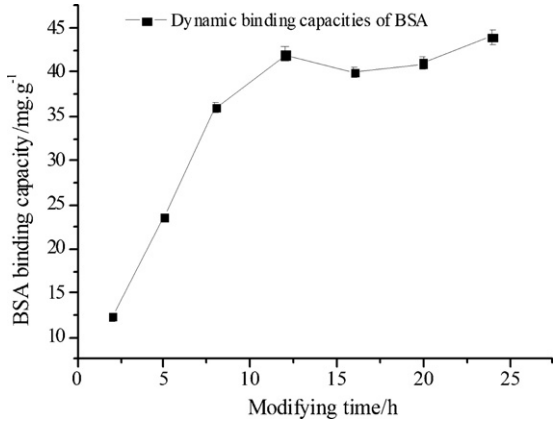

Fig. 3. The dynamic binding capacities of monoliths functionalized with PEI (30 kDa) for different modifying times. Modifying conditions: 10% (wt%) PEI (30 kDa) in water, modifying temperature 60 °C.

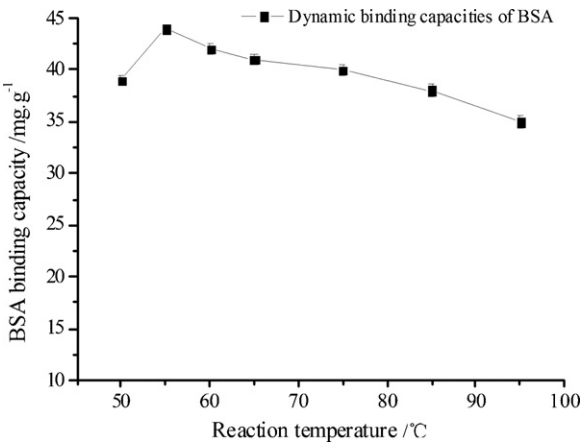

Fig. 4. The influence of modifying temperatures on the binding capacities of PEI (30 kDa) modified monoliths. Modifying conditions: 10% (wt%) PEI (30 kDa) in water, modifying time 12 h.

12–24 h, there is no obvious increase of the BSA binding capacity, which means the modifying reaction basically reaches equilibrium at 12 h. In order to save time, 12 h is chosen as the modifying time for monolith.

3.1.4. Effects of modifying temperature

The modifying temperature also affects the ring-opening reaction of the epoxy groups, which influences the property of the monolith. As indicated in Fig. 4, the binding capacity of BSA slightly decreases with the increase of temperatures after 55 °C. The influences of modifying temperature might be reflected from two aspects. For the poly(GMA-co-EDMA) skeleton, the epoxy groups link with the polymer skeleton through ester groups. The comparatively strong alkalescence of PEI can hydrolyze part of the ester groups. With the increase of temperatures, the hydrolysis rate of ester groups increases, resulting in part of the PEI ligands rupturing from the polymer skeleton. Consequently, the ligand density decreases. Meanwhile, with the increase of modifying temperature, the PEI coupling speed on the polymer skeleton increases, bringing large ligand density. Fig. 4 shows the integrated results of these two aspects. In the following experiments, 55 °C is chosen as the modifying temperature.

3.2. One-step fast separation and purification of YLLIP2

PEI is a weakly basic anion exchanger. Both the ternary and duality amine functionalities in polymer brush can afford weak ion-exchange property. Anion-exchange chromatography using PEI modified monolith allowed separation of the lipase from the colored components and most of other extracellular proteins. After optimization of the salt concentrations, 10 mM Tris–HCl buffer (pH 7.6) was demonstrated to be the best adsorption solution and 1 M NaCl gradient solution was the best elution. The lipase activity was bound to the monolith. After eluting an unbound fraction without activity, the lipase activity was eluted with 1 M NaCl gradient elution. Under the optimized preparative conditions, PEI 30 kDa with the concentration of 10% (v/v) was applied for the modification of the monolith at 55 °C for 12 h. Fig. 5 shows the direct separation of crude lipase on the optimized monolith with 1 M NaCl gradient elution at the flow rate of 1 mL min^{-1}. The separation of four isoforms can be achieved at the flow rate of 1 mL min^{-1} within 40 min.

The four isoforms, named isoform A, isoform B, isoform C and isoform D were separately collected and determined the activity, recovery and protein purification fold. As shown in Table 2, after one-single step purification, the lipase was efficiently puri-

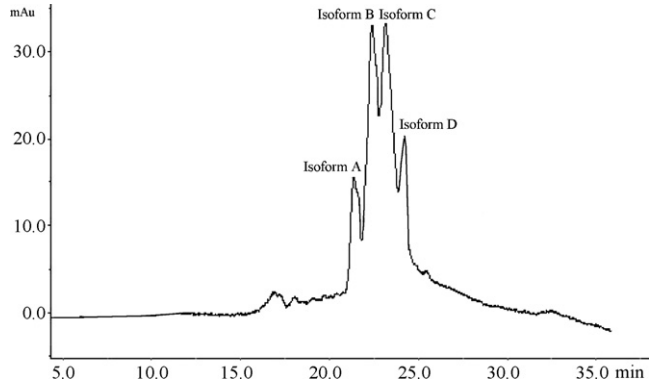

Fig. 5. One-step separation chromatogram of the crude lipase on the monolithic column (100 mm × 4.6 mm i.d.) modified by PEI 30 kDa water solution with a concentration of 10% (v/v). NaCl gradient from 0 M to 1.0 M in 7 mL at flow rate of l.0 mL min^{-1} was used for separation.

Table 2
The results of lipase purification on PEI (30 kDa) functionalized monolith.

Characters	Crude lipase	Isoform A	Isoform B	Isoform C	Isoform D
Total activity ($\times 10^3$ U)	94.3	3.30	18.7	15.7	11.6
Total protein (mg)	109	0.960	5.40	6.91	3.00
Specific activity ($\times 10^3$ U mg^{-1})	0.863	3.48	3.47	2.27	3.86
Purification fold	–	4.00	4.00	2.60	4.50
Yield (%)	52.2 (total)	3.50	19.8	16.6	12.3

fied with very high specific activities for each isoform. Analysis of the activity peak by non-denaturing PAGE shows a homogenous purified YLLIP2 with molecular masses of around 38 kDa and the band contains hardly any contaminants (Fig. 6). The differences of the four isoforms are further confirmed by MALDI-TOF mass analysis, which indicates molecular masses of 36.648 ± 36, 37.839 ± 33, 38.236 ± 31 and 38.795 ± 96 Da for purified isoforms denoted isoform A, isoform B, isoform C and isoform D, respectively. These masses are comparatively higher than those of the YLLIP2 reported in the literature [12]. The isoforms are the protein encoded by the same gene.

Although, the separation result is not as good as reference [12,18], PEI modified monoliths have great potential for one-single step separation and purification of crude lipases. Aloulou, et al. [12] adopted two purification steps, gel filtration chromatography performed on a Superdex 200 HR 26/60 column and anion-exchange chromatography performed on a Mono Q 5/50 GL column. Likewise, Fu et al. [18] used two ion-exchange chromatography steps on Q Sepharose column and Mono Q 5/50 GL column. To our knowledge, high performance Mono Q 5/50 GL column used in these two references is a highly efficient ion exchange chromatography matrix. However, these columns are a little pricy and cannot afford direct crude lipase loading due to the dramatic backpressure increase during the sample loading. Consequently, a normal performance chromatography step is needed for the preliminary purification of the crude lipase before loading on the Mono Q 5/50 GL columns. The PEI modified monolith can basically obtain four pure isoforms in one-single step without losing separation resolution and efficiency. In addition, thanks to the good mass transfer of the monolithic matrix, the monoliths can afford direct crude lipase loading without increasing too much backpressure. The preparation of the PEI modified monolith is of low-cost. After scaling up the preparation, the monolith can afford more samples loading in one-single step. Furthermore, PEI has a polymer chain. Varying the molecular mass of PEIs can change the length and then the flexibility of the side chain. Consequently, by changing the molecular mass of PEI modifiers, a variety of monoliths can be obtained versatile for different biomolecules.

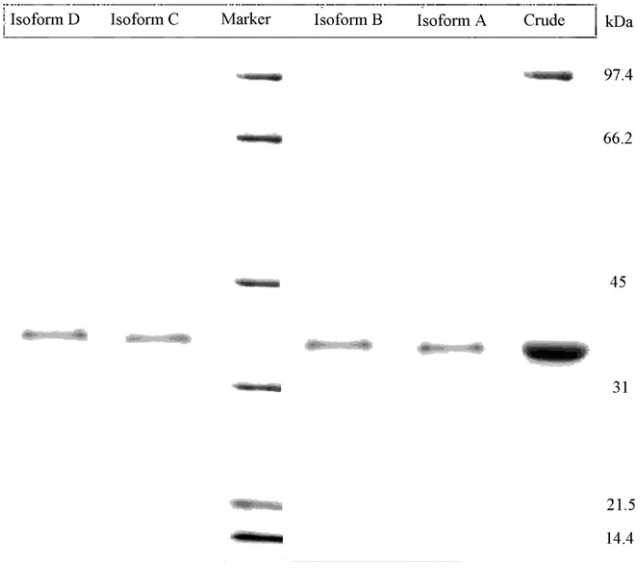

Fig. 6. Non-denaturing PAGE analysis of lipase.

4. Conclusions

The extracellular lipase YLLIP2 crude extract is successfully separated and purified with one-single step anion-exchange chromatography on PEI modified monolithic columns. The lipase is efficiently purified to be four isoforms with very high specific activities for each isoform. This method had obvious priority compared to multi-steps chromatography. It explored the potential application of PEI functionalized monolith for complex systems.

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