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Refolding, purification and characterization of an organic solvent-tolerant lipase from *Serratia marcescens* ECU1010

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ABSTRACT

Expression of recombinant proteins as inclusion bodies in bacteria is one of the most efficient ways to produce cloned proteins, as long as the inclusion bodies can be successfully refolded. In this study, the different parameters were investigated and optimized on the refolding of denatured lipase. The maximum lipase activity of 5000 U/L was obtained after incubation of denatured enzyme in a refolding buffer containing 20 mM Tris–HCl (pH 7.0), 1 mM Ca^{2+} at 20 °C. Then, the refolded lipase was purified to homogeneity by anion exchange chromatography. The purified refolded lipase was stable in broad ranges of temperatures and pH values, as well as in a series of water-miscible organic solvents. In addition, some water-immiscible organic solvents, such as petroleum ether and isopropyl ether, could reduce the polarity and increase the nonpolarity of the refolding system. The results of Fourier transform infrared (FT-IR) microspectroscopy were the first to confirm that lipase refolding could be further improved in the presence of organic solvents. The purified refolded lipase could enantioselectively hydrolyze *trans*-3-(4-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM]. These features render the lipase attraction for biotechnological applications in the field of organic synthesis and pharmaceutical industry.

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1. Introduction

Lipases (EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils. These enzymes play important roles in the pharmaceutical and fine chemical industries as well as in other industrial areas such as detergents, oil-fats and leather [1–3]. Among many successful examples of lipase application, the lipase from *Serratia marcescens* is well known in the pharmaceutical industry for its excellent enantioselectivity in the biocatalytic hydrolysis of *trans*-3-(4-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM] to produce (2*R*, 3*S*)-3-(4-methoxyphenyl) glycidic acid methyl ester [(−)-MPGM], a key intermediate for the synthesis of diltiazem hydrochloride [4–6].

The extracellular lipase produced by *S. marcescens* ECU1010 has been demonstrated to be promising for the production of enantiopure 3-phenylglycidate [4]. Recently, Bae et al. [7] reported that a lipase from *S. marcescens* ES-2 was used for kinetic resolution of racemic flurbiprofen, giving an optically pure (*S*)-flurbiprofen (98.5% *ee*) with a very high enantioselectivtiy (*E* = 332). Jaeger et al. [8] tried to use the lipase from *S. marcescens* SM6 for the kinetic resolution of other racemic esters such as isopropylideneglycerol acetate in organic solvent but the reactions were failed. In previous work, the lipase gene (*lip*A) was successfully cloned and overexpressed as soluble form in *Escherichia coli*. After optimization, the maximum lipase activity reached 5000–6000 U/L and this recombinant lipase could enantioselectively hydrolyze (*S*)-ketoprofen esters into (*S*)-ketoprofen with high enantiomeric excess. Nonetheless, the soluble fraction (about 30% of the total proteins) was far fewer than the insoluble fraction presented as inactive inclusion bodies [9], which severely restricted the industrial applications. In this work, we have performed successfully refolding and improved the final yield of active lipase. Zhao et al. [10] have demonstrated that the lipase from *S. marcescens* was quite stable in the presence of various organic solvents. However, the mechanism was still not fully clarified.

In the present study, recombinant lipase inclusion bodies were solubilized and active recombinant lipase protein was obtained by the method of rapid dilution based on the optimization of the refolding process. Afterwards, the refolded lipase was purified by a single step anion-exchange chromatography and characterized by different physicochemical methods. Finally, the activation

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FT-IR, Fourier transform infrared; IPTG, isopryl-β-thiogalactopyranoside; KPB, potassium phosphate buffer; (−)-MPGM, (2*R*, 3*S*)-3-(4-methoxyphenyl) glycidic acid methyl ester; (±)-MPGM, *trans*-3-(4-methoxyphenyl) glycidic acid methyl ester; *p*NPA, *p*-nitrophenyl acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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mechanism of the enzyme in some organic solvents was investigated by Fourier transform infrared (FT-IR) microspectroscopy.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The expression plasmid pET-24a(+) (Novagen, Madison, USA) with the lipase gene from *S. marcescens* ECU1010 was constructed by Xu et al. [6]. *E. coli* BL21 (DE3) was used as a host for lipase protein expression. All other chemicals were obtained commercially and were of analytical grade.

Strains were grown regularly in Luria-Bertani (LB) medium containing 50 μg/mL kanamycin at 37 °C with shaking at 160 rpm. When the optical density (OD_{600}) reached about 1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After induction for a further 4 h, cells were harvested by centrifugation at 4000 × *g* for 30 min.

2.2. Preparation and solubilization of lipase inclusion bodies

Cells were collected from four 500-mL flasks containing 200 mL of culture medium, suspended in 20 mL 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, and disrupted by an ultrasonic disruptor at 200 W intermittently in an ice bath. Disrupted cells were centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant and the precipitate were collected as soluble and insoluble fractions, respectively. Subsequently, the cell pellets containing the insoluble fraction were washed with 5 volumes of 50 mM Tris–HCl (pH 8.0), 20 mM EDTA, 100 mM NaCl, 2% (v/v) Triton X-100, at room temperature for 30 min, followed by 10 volumes of the same buffer without Triton X-100 at room temperature for 30 min, to remove some of the impurities from the inclusion bodies. The pellets were then dissolved in 10 mL of 50 mM Tris–HCl (pH 8.0) containing 8 M urea and incubated at room temperature for 3 h with mild stirring. The amount of solubilized protein was determined by measuring the protein concentration of the supernatant.

2.3. Optimization of refolding conditions

Refolding of the purified lipase was conducted by rapid dilution of the protein mixture by 10-fold, using freshly prepared refolding buffer. In order to determine the optimal conditions for refolding, the refolding parameters covering Ca^{2+} and ionic strength were investigated and the refolding yield was studied after 12 h of incubation at 30 °C. To study the role of temperature in the refolding process, denatured lipase was added to the refolding buffer at different temperatures (4–37 °C) for 12 h. Then the refolded protein was assayed for lipase activity. To determine the optimal pH, denatured lipase was diluted in the refolding buffer with different pH values ranging from 6.0 to 8.0.

2.4. Purification of the refolded lipase

Purification of lipase has been carried out by ion exchange chromatography as reported in literature [11,12]. The refolded lipase was applied to a DEAE Sepharose Fast Flow (DEAE-FF) column (⌀ 2 cm × 15 cm, Pharmacia, Sweden), which was pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.0). Target protein was finally eluted with a linear gradient of 0–1.0 M NaCl in the same buffer at 0.5 mL/min. Fractions of 2 mL each were collected and checked for the enzyme activity. Fractions showing lipase activity were pooled and concentrated before characterization. The samples were then stored at 4 °C until used. The purity of the refolded lipase was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie brilliant blue [13].

2.5. Activity and protein assays

Lipase was assayed using *p*-nitrophenyl acetate (*p*NPA) as a substrate. Lipase or blank solution (100 μl) was added to 2.87 mL of 100 mM potassium phosphate buffer (KPB, pH 7.5). After pre-incubation at 30 °C for 3 min, the reaction was initiated by quick mixing of the reaction mixture with 1 mM *p*NPA solution in dimethyl sulfoxide (DMSO). The variation in absorbance at 405 nm was then recorded. One unit of lipase activity was defined as the amount of enzyme that released 1.0 μmol of *p*-nitrophenol per minute under such conditions [4–6]. Protein content was estimated by Bradford dye binding assay using bovine serum albumin (BSA) as a standard [14]. The protein eluted out of the column during chromatography was detected at 280 nm.

2.6. Enzyme characterization

The stability of the refolded lipase to temperature was investigated by detecting the residual activity after incubating the purified lipase at a temperature range of 30–60 °C for 20 min, 1, 2 and 4 h. The effect of pH on lipase stability was determined by detecting the residual activity after incubating the purified lipase in the buffers with different pH values at 25 °C for 2 h. The effect of various organic solvents (50%, v/v) on the lipase activity was investigated as follows [15]: the reaction mixture was incubated for 4 h at 25 °C with shaking (160 rpm). The residual activity was assayed under the standard condition and expressed as a percentage of the lipase activity in 100 mM KPB (pH 7.5) without any organic solvent.

2.7. Fourier transform infrared (FT-IR) microspectroscopy

Lipase solution (refolded lipase or purified lipase) was deposited onto a BaF_2 window and dried at 22 °C. FT-IR absorption spectra from 400 to 4000 cm^{-1} were acquired in the transmission mode by coupling the Nicolet 6700 infrared microscope equipped with a nitrogen cooled MCT detector (narrow band, 250 μm) to a FTS 40 A spectrometer (Digilab, USA) at 4 cm^{-1} resolution, 20 kHz speed, 256 scan co-additions, and triangular apodization. Absorption spectra with a low noise level were obtained by setting the microscope aperture at approximately 100 μm × 100 μm [16–18]. The background spectrum was collected before each measurement, and no baseline correction was required for the spectra. Spectra were only corrected for possible residual water vapor.

A second derivative analysis of the spectra on the Amide I region was performed after a 15-point smoothing by the Savitzky–Golay method (3rd polynomial, 13 smoothing points) using the OMNIC software (Thermo Nicolet, USA).

2.8. Chiral selectivity of the refolded lipase

The chiral selectivity of the refolded lipase was investigated in a toluene:water (1:1) two-phase system using (±)-MPGM as substrate. A total of 5 mL of 100 mM KPB (pH 7.5) containing the refolded lipase (10 U/mL) was added to 5 mL of toluene solution with 100 mM (±)-MPGM. The reaction in the biphasic system was carried out at 30 °C with shaking at 160 rpm in 100-mL flask equipped with tight plugs for 4 h. The concentrations of optical isomers were determined by HPLC with a chiral column (Chiralcel OJ, 25 cm × Φ 4.6 cm, Daicel Chemical Industries, Tokyo, Japan), with elution by *n*-hexane:isopropanol (60:40, v/v; 0.8 mL/min) and detection at 254 nm [6].

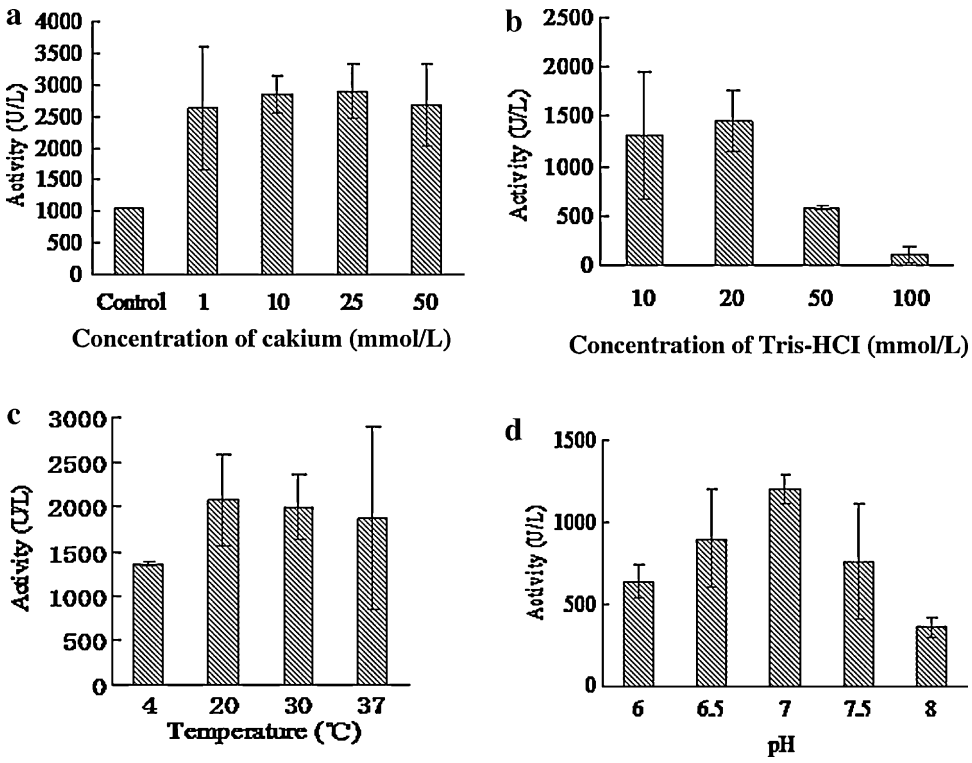

Fig. 1. (a) Effect of Ca^{2+} on lipase refolding. A total of 50 mM Tris–HCl pH 8.0 as the refolding buffer with the presence of Ca^{2+} at different concentrations. (b) Effect of concentration of refolding buffer on lipase refolding. Different concentrations of Tris–HCl pH 8.0 as the refolding buffer. (c) Effect of temperature on lipase refolding. A total of 20 mM Tris–HCl pH 8.0 as the refolding buffer at different temperatures. (d) Effect of pH on lipase refolding. A total of 20 mM Tris–HCl with different pH values of the refolding buffer. All assays were performed in triplicate.

3. Results and discussion

3.1. The optimization of the conditions of lipase refolding

Active enzyme is known to be achieved from inclusion bodies by using *in vitro* refolding techniques. Refolding protocols are usually designed based on parameters such as temperature, pH and ionic strength, as well as protein concentration and suppressor of aggregation [19]. In this study, the effects of significant parameters including Ca^{2+}, temperature, pH and ionic strength on lipase refolding were investigated by single factor experiment.

The Ca^{2+} ion acts as an activator of lipase and plays an important stabilizing role in the conformation of the protein [20–24]. To examine whether the activity of the lipase was also enhanced in the presence of Ca^{2+}, refolding was performed in the refolding buffer with the presence of Ca^{2+} at different concentrations. The result indicated that lipase activity increased more than 2-fold in the presence of Ca^{2+}. Increasing the concentration of Ca^{2+} up to 50 mM had no significant effect on lipase refolding (Fig. 1a). It has been reported that the lipases from *Burkholderia glumae* [25] and *Staphylococcus hyicus* [26] contain a Ca^{2+} binding site, which is formed by two conserved aspartic acid residues, near the active-site, and binding of Ca^{2+} to this site dramatically enhanced the activities of these enzymes. The lipase from *S. marcescens* possessed a similar structure [24]. The ionic strength of the refolding buffer was also one of the key factors in lipase refolding. By increasing the ionic strength of the refolding buffer, the hydrophobic interactions are enhanced [27]. As shown in Fig. 1b, the activity of lipase increased when the concentration of Tris–HCl buffer was increased from 10 to 20 mM; however at higher concentrations it was relatively low. Although the difference between 10 and 20 mM was not statistically significant, the maximum activity was obtained when the refolding was performed in 20 mM Tris–HCl buffer. Based on these results, it was inferred that less aggregation and misfolding would occur in the presence of Ca^{2+} in the refolding buffer with appropriate ionic strength. Moreover increasing the concentration of lipase in the refolding buffer increased the refolding efficiency which indicated that protein concentration was also an important parameter affecting refolding process (data not shown). In addition, the refolding process was allowed to take place for 12 h at 4, 20, 30 and 37 °C by simple dilution of soluble lipase (0.4 mg/mL) in the refolding buffer containing Ca^{2+} (Fig. 1c). The results revealed that, the highest refolding yield achieved at 20 °C. Over the range of pH explored, the highest refolding yield was achieved in pH 7.0 (Fig. 1d). From these results we concluded that Ca^{2+} and ionic strength were the most important variables that influenced the final refolding yield. In this study, 1 mM Ca^{2+} and 20 mM Tris–HCl buffer have been determined as the best condition for lipase refolding. Under the optimal conditions, the maximum lipase activity reached 5000 U/L, which was consistent with the results reported by Long et al. [9].

3.2. Purification of the refolded lipase

Although high activity was obtained after refolding in the 20 mM Tris 7.0, with 1 mM Ca^{2+}, electrophoretic analysis indicated a small amount of impurities in the refolded proteins (data not shown). Therefore in order to separate the impurities, further purification was done using DEAE-FF ion-exchange column chromatography and 20 mM Tris 7.0 as the loading buffer. The bound proteins were eluted with linear gradient of 0–1.0 M NaCl. The contents of the preload protein sample in the eluted fractions were shown as two peaks at 280 nm absorbance (Fig. 2a) and the purified samples were analyzed by SDS-PAGE (Fig. 2b). The first elution peak showing high activity was defined as P (I), while the second one showing low activity was defined as P (II). Coomassie brilliant blue staining revealed that P (I) and P (II) were both lipase (Fig. 2b). Main difference between P (I) and P (II) was that the specific activity of P (I) was low, while that of P (II) was high (data not shown). Compared with

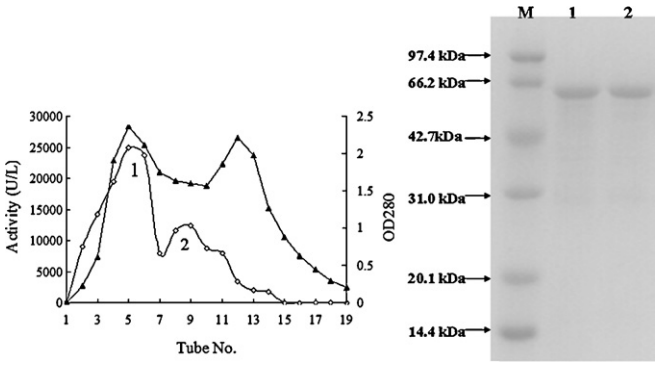

Fig. 2. (a) Elution profile of refolded lipase in DEAE-FF column. Bound proteins were eluted with a linear gradient of 0–1.0 M NaCl. Protein was detected at 280 nm (▲) and lipase activity (◊) by lipase assay. (1) The peak of polymeric lipase P (I); (2) the peak of monomeric lipase P (II). (b) Ion-exchange chromatography analysis by SDS-PAGE. Lane M, molecular mass marker protein; lane 1, polymeric lipase P (I); lane 2, monomeric lipase P (II).

the band of native lipase dimer, with a reported molecular mass 140 kDa [10] and a monomer molecular mass of 65 kDa, P (I) was a polymeric lipase, and P (II) appeared to be a monomeric lipase. Therefore, it was inferred that the native lipase is a dimer with two homologous subunits. The refolded lipase was finally purified to 1.4-fold with 83.7% yields by a single step of DEAE-Sepharose ion exchange chromatography (Table 1). This ion exchange purification step was enough to produce substantial amounts of the purified lipase.

In order to compare the differences on secondary structure between the polymeric and monomeric lipase, FT-IR spectroscopy was efficiently used for structural analysis. It has been shown that the analysis of the Amide I band components enabled the detection of protein aggregations [16–18]. FT-IR second derivative of the Amide I band showed six principle components from 1683 to 1623 cm^{-1}, which can be assigned to the γ-turn, α-helix and unordered, as well as β-sheet and protein aggregations [28]. These derivative spectra are reported in the region of the Amide I band from 1700 to 1600 cm^{-1} in Fig. 3a. The resulting spectra displayed important differences, particularly in the Amide I region of the unordered absorption. In the case of P (I), which was the low specific activity polymeric lipase, there was no evidence of unordered absorption at 1650 cm^{-1} based on the direct inspection of the second derivative spectrum. Accordingly, the unordered peak at 1650 cm^{-1} was one of the typical structures of monomeric lipase. The contents of the γ-turn (1683 cm^{-1}), α-helix (1660 cm^{-1}) and β-sheet (1641 cm^{-1}) of the polymeric lipase were lower than those of the monomeric lipase, while the content of aggregation (1623 cm^{-1}) was higher than monomeric lipase. From these results we concluded that some incorrect secondary structures in the process of refolding caused the formation of polymeric lipase. It was also consistent with the report that the peak of the second derivative spectrum leaned more toward higher wavenumbers, while the second structure was more similar to the natural structure [28].

Table 1
Purification of the refolded lipase from *S. marcescens* ECU1010.

Purification step	Total activity[a] (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Refolded lipase	1410	100	14.1	1	100
DEAE-Sepharose	1180	61	19.3	1.4	83.7

[a] The activity was assayed using *p*NPA as substrate.

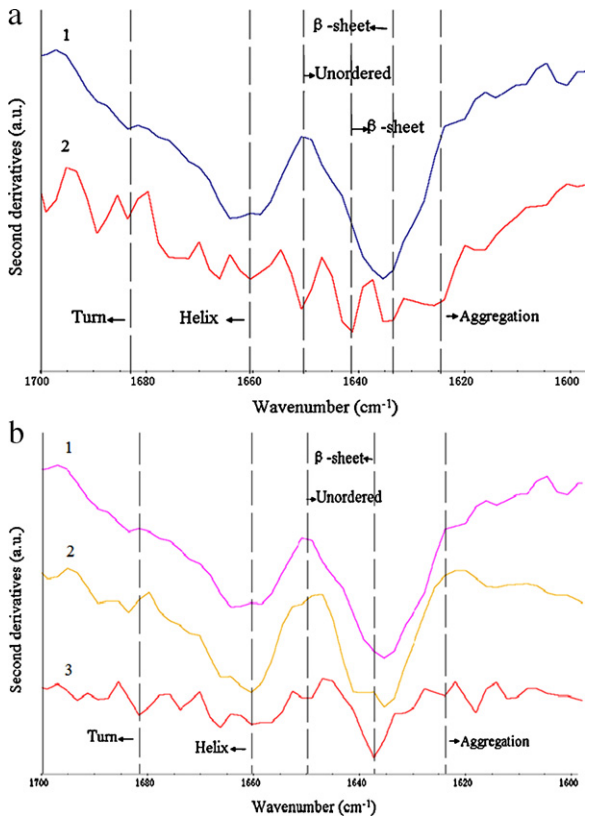

Fig. 3. (a) FT-IR second derivative spectra of polymeric lipase P (I) (1) and monomeric lipase P (II) (2). (b) FT-IR second derivative spectra of polymeric lipase mixed with organic solvents. (1) Polymeric lipase; (2) polymeric lipase mixed with 50% (v/v) isopropyl ether; (3) polymeric lipase mixed with 50% (v/v) petroleum ether.

3.3. Characterization of the refolded lipase

It is known that variation of solution pH strongly influences the ionic environment of an enzyme, thus affecting its interaction with the substrate and enzymatic activity. Therefore, the effect of pH on the refolded lipase stability was investigated by incubating the enzyme in the pH range 3.0–12.0 for 2 h at 25 °C, with residual activity determined as previously described. As shown in Fig. 4a, the refolded lipase showed the maximum activity at pH 7.0 and remained over 40% of its initial activity at pH 5.0–9.0. However, the residual activity decreased sharply at pH over 9.0. At 30 and 40 °C, after 4 h incubation, the enzyme remained about 80–100% residual activities compared with the initial activity (Fig. 4b). When treated for 20 min at 60 °C, the enzyme was almost deactivated. These results were consistent with the other alkaline lipases from the strains of *Pseudomonas*, *Acinetobacter* and *Bacillus subtilis* [29–31].

3.4. Tolerance of the refolded lipase against organic solvents

The stability in organic solvents is an important characteristic of lipases. It can determine whether the enzyme can be used to catalyze synthetic reactions and also to predict which solvent would be better to perform the reaction. The effect of the organic solvent depends on the nature of both enzyme and solvent [32]. In this study, the tolerance of the polymeric lipase P (I) was studied against some water miscible and immiscible solvents. As shown in Table 2, the refolded lipase was activated and displayed high stability in water-immiscible organic solvents with a residual activity of 300% for 50% (v/v) isopropyl ether and 183% for 50% (v/v) petroleum ether. On the contrary, this phenomenon was not observed for

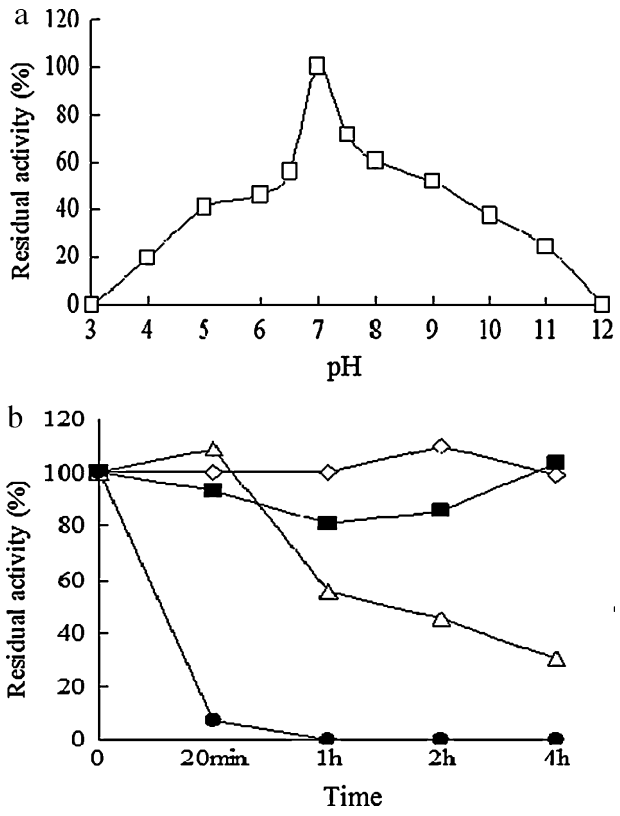

Fig. 4. (a) Effect of pH on stability of refolded lipase. Purified refolded lipase was incubated at pH 3.0–12.0 for 2 h at 25 °C, and residual activity determined as described in Section 2. (b) Effect of temperature on stability of refolded lipase. Assay was performed at 30 °C (◊), 40 °C (■), 50 °C (△) and 60 °C (●) and residual activity determined from 0 to 4 h.

monomeric lipase P (II) (data not shown). These results were in agreement with Zaks and Klibanov [33]. It was proposed that residues of the hydrophobic solvent, which may be transferred to the assay system with the enzyme after the pre-incubation step, keeps it in the open conformation and therefore the lid of the enzyme does not block the active site crevice, keeping a flexible conformation and therefore increasing the activity [34]. In addition, no drastic decreases in residual activity were observed after incubation for 12 h at 10% (v/v) of water-miscible organic solvents such as glycerin and acetone. DMSO, which is widely used to dissolve proteins to a certain extent, slightly activated the enzyme at 10% concentration. It was believed that water-miscible organic solvents strip water from the enzymes, leading to the unfolding of the molecule with exposure of the inner hydrophobic residues and that this denaturation occurs at a much faster rate than in a pure aqueous system [35]. The same behavior was found for the lipases from *Penicillium simplicissimum* [36] and *Mucor* sp. [37].

Although the refolded lipase showed distinct stability against various organic solvents, the mechanism of the increased activity of polymeric lipase was further investigated after treatment with isopropyl ether or petroleum ether. The results of FT-IR revealed the structural transformation of the protein. The unordered peak at 1650 cm^{-1} was observed when polymeric lipase was mixed with isopropyl ether or petroleum ether. In addition, the contents of β-sheet at 1641 cm^{-1} and α-helix at 1660 cm^{-1} were increased, while the content of aggregation at 1623 cm^{-1} was decreased (Fig. 3b). This indicates that the correct structure formed again when polymeric lipase was mixed with isopropyl ether or petroleum ether. From the analysis above, we considered that both isopropyl ether and petroleum ether could reduce the polarity and increase the nonpolarity of the refolding system. It was the first to confirm that lipase refolding could be further improved in the presence of organic solvents.

3.5. Chiral selectivity of the refolded lipase

As it may be useful for kinetic resolution of chiral compounds, we tried to use this refolded lipase for enantioselective hydrolysis of (±)-MPGM. In a standard experiment, 5 mL of the refolded lipase (ca. 10 U/mL, as measured by lipase assay) solution was added to 5 mL of toluene solution with 100 mmol/L (±)-MPGM. The concentrations of (+)-MPGM and (−)-MPGM were almost equal in the 5-mL toluene solution of 100 mmol/L (±)-MPGM prior to the reaction. When the reaction in the biphasic system was carried out for 4 h, the refolded lipase enantioselectively catalyzed the hydrolysis of (±)-MPGM to (−)-MPGM (Supplemental Fig. S1). The reaction proceeded at a substrate concentration of 100 mM, resulting in a highly pure product, (−)-MPGM, with high enantiomeric excess (99%) and *E*-value (>100), which is known as a key intermediate for production of an important antihypertensive agent diltiazem. These results suggested that the refolded lipase presented the same activity toward (±)-MPGM, in contrast to the wild-type lipase from *S. marcescens* ECU1010 [6].

Table 2
Stability of purified refolded lipase against various solvents.[a]

Solvent	Residual activity (%)[b]		
	10% (v/v) concentration	25% (v/v) concentration	50% (v/v) concentration
DMSO	118 ± 3	99 ± 10	0
Ethanol	73 ± 3	36 ± 5	0
Glycerol	81 ± 4	83 ± 6	79 ± 3
Acetone	103 ± 6	89 ± 1	0
Isopropanol	160 ± 7	117 ± 12	11 ± 2
Isopropyl ether	107 ± 9	160 ± 17	300 ± 9
Petroleum ether	103 ± 10	119 ± 2	183 ± 15

[a] The purified refolded lipase was incubated in various solvents at 30 °C for 12 h.
[b] Residual activities were measured by the standard assay with *p*NPA.

4. Conclusion

In conclusion, the high tolerance against organic solvent makes the *S. marcescens* ECU1010 lipase a very attractive enzyme for potential application in industry, particularly in the field of biocatalytic resolution to produce enantiopure building blocks for chiral pharmaceuticals or agrochemicals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.04.016.

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