Full Paper

Expression in *Pichia pastoris* and characterization of *Rhizomucor miehei* lipases containing a new propeptide region

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A large number of propeptide regions from various proteins have been identified which function as intramolecular chaperones and assist the folding of the respective functional domains. The same polypeptide can fold into an altered conformation because of a mutated intramolecular chaperone and can maintain the “memory” of the folding process (new physicochemical properties). Two new kinds of *Rhizomucor miehei* lipase (RML) were constructed by replacing its propeptide region with that from either *Rhizopus chinensis* lipase (RCL) or *Rhizopus oryzae* lipase (ROL). The enzymatic properties were also analyzed and compared between wild-type RML and the mutants. The results indicated that the same polypeptide can fold into different conformations because of changes in the propeptide region.

Key Words: enzyme property; intramolecular chaperone; propeptide replacing; protein folding; *Rhizomucor miehei* lipases

Abbreviations: RML, *Rhizomucor miehei* lipase; RCL, *Rhizopus chinensis* lipase; ROL, *Rhizopus oryzae* lipase; pro, propeptide

Introduction

Propeptides, which are N-terminal or C-terminal domains of many proteins, are cleaved off in the protein maturation steps. They are usually located between the signal peptide and the functional region of a protein, and function as intramolecular chaperones (IMCs) that facili-

tate protein folding (Eder and Fersht, 1985; Ikemura et al., 1987; Inouye, 1991; Lee et al., 1991; Shinde and Inouye, 1993, 2000; Vandenhazel et al., 1993). A growing body of evidence has revealed that these IMCs play an important role in protein folding both *in vivo* and *in vitro* (Chen and Inouye, 2008). Studies of α -lytic protease suggest that the propeptide region folds independently from the protease domain and that the functional region of the protein cannot form the native structure without the propeptide region (Anderson et al., 1999). Shinde et al. (1997) demonstrated that the mutation I48V in the propeptide of Subtilisin E can cause the same polypeptide to fold into different conformations, each with distinct thermostability and substrate specificities. Their results indicate that the same polypeptide can fold into an altered conformation because of a mutated IMC (Shinde et al., 1997). Increasing efforts have been devoted to the optimization of the protein function via a modification of propeptide regions.

Kakeshita et al. (2011) found that secretion, production, and activity of hIFN- α -2b with an AmyE propeptide region increased more than threefold in comparison with that without the AmyE propeptide region. The expression level of keratinase Sfp2 from *Streptomyces fradiae* was improved by site-directed mutagenesis of its N-terminal prosequence (Li et al., 2013). Carboxypeptidase Y (CPY) with a mutated prosequence was successfully displayed on the yeast cell surface. Measurement of the activity and kinetics indicated that the propeptide mutation altered the catalytic efficiency of mature CPY (mCPY) (Nagayama et al., 2012).

Rhizomucor miehei lipase (RML; GenBank accession No. A02536) is an enzyme particularly important for the food industry because of the ability to hydrolyze 1,3-position C(O)-O bonds in triglyceride esters (Rodrigues and

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Fernandez, 2010). After translation, RML contains 24 amino acid residues of the signal peptide and 70 amino acid residues of the propeptide region at the upstream of the functional region. RML is thought to cross the cytoplasmic membrane with the aid of the signal peptide after the active protein is produced. Subsequently, the propeptide region is removed by protease (Boel et al., 1988).

Wang et al. (2012) reported that the activity of RML was improved because of the crucial role of mutations in the propeptide. At the same time, their study showed that RML with glycosylation in its propeptide region has a lower enzymatic activity than RML without glycosylation in its propeptide region (Liu et al., 2014). These findings proved that the propeptide region has a strong influence on the characteristics of RML. In the present study, two new kinds of lipases from RML were constructed by replacing the propeptide region with that from *Rhizopus chinensis* lipase (RCL) and *Rhizopus oryzae* lipase (ROL). The two kinds of lipases with new propeptide regions were successfully expressed in *Pichia pastoris*. The enzyme activity and other characteristics were analyzed and compared between the wild-type propeptide and the mutants.

Materials and Methods

All of the restriction enzymes and DNA polymerase were purchased from Takara Shuzo Co., Ltd., and *Escherichia coli* DH5 α (DE3) cells, *P. pastoris* GS115 and the plasmid pPIC9K were purchased from Invitrogen.

T4 DNA ligase, and the BCA Protein Assay Kit were purchased from Sangon Biotechnology (Shanghai, China). 4-Nitrophenyl palmitate (pNPP) and trichloroacetic acid were purchased from Sigma. The filter that we used to concentrate the crude protein in the medium was purchased from Millipore. Yeast cells transformation were taken by Eppendorf Electroporator 2510 (Germany). Enzyme purification was performed using the NGC Chromatography System (Bio-Rad, China) equipped with a diethylaminoethanol (DEAE) Sepharose Fast Flow column, anion exchange column, and SRT-SEC column. Amberlite IRA-93 was purchased from Cangzhou Co., Ltd. (Hebei, China). Samples were boiled in 120 mM Tris-Cl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 3% glycerol, and 1% β -mercaptoethanol (β -ME) for 10 min. Samples were centrifuged at $10,000 \times g$ for 5 min, and supernatants subjected to a 12% SDS-polyacrylamide gel electrophoresis (PAGE) with 0.1% SDS in a vertical slab gel apparatus (Laemmli, 1970).

The YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) was used for cultivation of *P. pastoris* GS115, and all transformants were selected on plates with minimal dextrose (MD) medium (1.34% yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, and 2% dextrose, 1.5% agar). The BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 100 mM potassium phosphate buffer, pH 6, and 1% glycerol) and BMMY medium (BMGY with 0.5% methanol instead of 1% glycerol) were used for expression of the recombinant *P. pastoris* proteins. All other chemicals were of analytical grade.

Cloning of the RML gene, construction of the lipase ex-

pression vectors, and transformation. The RML gene was synthesized by Sangon Biotechnology (Shanghai, China) after optimization according to the codon bias of *P. pastoris*; the optimization was based on the sequence of the lipase from *R. miehei* (GenBank accession No. A02536). The RML gene with the wild-type propeptide sequence was named wildpro-RML (GenBank accession No. KP164599). The gene was cloned into the pUC18T vector, named pUC18T-wildproRML. All the sequencing was performed by Beijing Genomics Institution. The prosequences from RCL and ROL were synthesized in the same way after optimization and were cloned into the pUC18T vector, named pUC18T-RCLpro and pUC18T-ROLpro.

The wildpro-RML was ligated into the respective sites of pPIC9K resulting in the pPIC9K-wildproRML, where the gene is under control of the methanol-inducible alcohol oxidase 1 promoter (PAOX1) and is fused in-frame with the α -factor secretion signal peptide of *S. cerevisiae*.

For construction of the new RML lipase gene (with the propeptide region from *R. chinensis* lipase), the primers RCL-1 (5'-GGGTACGTAATGGTTCCTGTTG) and RCL-2 (5'-ATCGATTGACATGCTGGGAGCAGT) were used to amplify the prosequence from pUC18T-RCLpro; primers RML-MatureF-1 (5'-ACTGCTCC CAG CATGTC-AATCGAT) and RML-MatureF-0 (5'-GGAATTCTT-ACGTGCACAACC) were used to amplify the mature-lipase gene fragment from pUC18T-proRML. Then, these two PCR fragments were ligated by overlap extension PCR to generate the new lipase gene RCLpro-RML (GenBank accession No. KP164600).

For construction of another new RML lipase gene (with the prosequence from ROL), primers ROL-1 (5'-GGGTACGTAATGGTGCCTGTAT) and ROL-2 (5'-ATCGATTGACATGGCAGAATTAGT) were used to amplify the prosequence from pUC18T-ROLpro; primers RML-MatureF-2 (5'-ACTGCTCCCAGCATGTC AATCGAT) and RML-MatureF-0 (5'-GGAATTCTTACGTGCACAACC) were used to amplify the mature-lipase gene fragment from pUC18T-proRML. These two PCR fragments were then ligated by overlap extension PCR to generate the new lipase gene ROLpro-RML (GenBank accession No. KP164601).

During amplification of the new RML lipase gene, restriction sites *Sna*B I (TACGTA) and *Eco*R I (GAATTC) were incorporated into the forward and reverse primer sequence, respectively. The PCR products (from amplification of RCLpro-RML and ROLpro-RML) were digested with *Sna*B I and *Eco*R I, and these DNA fragments were ligated into the respective sites of pPIC9K resulting in the constructs pPIC9K-RCLpro-RML and pPIC9K-ROLpro-RML, respectively.

The above-mentioned recombinant plasmids (pPIC9K-wildproRML, pPIC9K-RCLpro-RML, and pPIC9K-ROLpro-RML) were transformed into *P. pastoris* GS115 cells as *Bgl* II-linearized plasmids by electroporation, and the selection of His⁺ transformants was performed on the MD medium (Invitrogen BV).

Expression of the lipases in P. pastoris. A 5-ml overnight culture was transferred into 50 ml of the BMGY medium for large-scale cultivation until the optical density at 600

RCL	VFVAGHKG.SVKATNGIL.FQLPPLISSRCTPSPHPETTGDPDAEAYYINKSVQWYQAHGNNYALIKRDTETVGGMLID	78
ROL	VFVSGKSGSSNTAVSASQNALPPLISSRCALPSNKGSKSLCAEPYNMQKNTWYESHGNNLTSGKRDNLVGGMLID	80
RML	VEIKRQSN.....STV...SLPPLIPSRITSPPSSPSTTDEPAPAM.....SRNGPLP...SVETRYGMALN	58
RCL	LPENFPPIPATSTAPS	94
ROL	LPSDAPPISLSSSTNS	96
RML	ATSYEDSVVQA.....	69

Fig. 1. Propeptide alignment of amino acid sequences from RCL, ROL and RML.

nm (OD_{600}) reached 2–5. The supernatant was removed by centrifugation at 2000 rpm for 5 min, and the cell pellet was resuspended to $OD_{600} = 1.0$ in 50 ml of the BMMY medium. Methanol (5 g/l) was added every 24 h. After the culture was shaken in a flask for 96 h, the supernatant was collected.

The *P. pastoris* His⁺ transformants were cultured in 25 ml of the buffered glycerol-complex medium (BMGY; 10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g/l YNB, 4×10^{-4} g/l biotin, 10 g/l glycerol), which was incubated by shaking at 28°C and 250 rpm in 250-ml glass flasks. When the cultures reached an OD_{600} of ~6, the cells were pelleted by centrifugation and resuspended in 20 ml of the buffered methanol-complex medium (BMMY; 10 g/l yeast extract, 20 g/l peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g/l YNB, 4×10^{-4} g/l biotin, 5 g/l methanol) to an OD_{600} of 1.0, and the culture was incubated with shaking at 28°C and 250 rpm in 100-ml glass flasks for 96 h. The cultures were supplemented with methanol (5 g/l) every 12 h to induce expression of the lipase. The culture was centrifuged, and the supernatant was collected for SDS-PAGE and the lipase activity assay.

The lipase activity assay. The commonly-used procedure to investigate lipase activity uses 4-Nitrophenyl palmitate (pNPP) and the release of *p*-nitrophenol, measured spectrophotometrically at 405 nm. Free lipases were dissolved in lipase assay buffer (50 mM Tris-HCl, pH 8.0), mixed with substrate solution (0.0625 mM pNPP, 0.1% Triton X-100 in lipase assay buffer), and reacted for 5 min before measuring OD_{405} with a kinetic microplate reader. Reactions were terminated by the addition of 20 μ l of 20% trichloroacetic acid solution. After centrifugation at $10,000 \times g$ for 1 min, a 200- μ l aliquot of the resultant supernatant was placed in a 96-well plate and measured. Product yields were determined from a standard curve prepared using *p*-nitrophenol as a standard. One unit of activity was defined as the amount of enzyme that released 1 μ mol of pNP per minute from pNPP under the assay conditions. Each sample was assayed in triplicate and the average value was determined.

Lipase purification and determination of protein concentration. The recombinant enzymes from the culture supernatant (after cultivation of the cells for 96 h) were purified as follows. The supernatant from the expression medium was concentrated and the buffer was replaced with 10 mM Tris-HCl buffer (pH 7.5) by ultrafiltration through a 10-kDa membrane (Millipore, USA). Then, the concentrated solution was loaded onto an SP-Sepharose column (Pharmacia; 1.6×20 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.5); the proteins were eluted with 0–0.5

M NaCl in the same buffer. The fractions containing lipase activity were pooled, concentrated, and subjected to chromatography on a Phenyl-Sepharose 6 FF column (Pharmacia; 1.6×20 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1.6 M ammonium sulfate. The lipase was then eluted with an ammonium sulfate concentration gradient from 1.6 to 0 M in 50 mM Tris-HCl buffer (pH 7.5); 4-ml fractions were collected at a flow rate of 0.8 ml/min. Purified lipases were stored at –20°C until use. The Bradford method was used to measure protein concentration in solutions. A 20% (v/v) solution of dye reagent was prepared with distilled water. A sample of 0.01 ml was added into 0.99 ml of Bradford solution. The test tubes were incubated at ambient temperature for 10 min. The quantity of protein in solution was determined by spectrophotometer (595 nm) using bovine serum albumin as the standard protein.

Effects of temperature and pH on the enzymatic activity. The optimal pH was determined by examining the activity of the enzymes at 40°C in the pH range 5.0–11.0. The optimal temperature was determined by measuring the enzymatic activity at the optimal pH at various temperatures (20–60°C). Each sample was assayed in triplicate and the average value was determined.

Effects of temperature and methanol on enzyme stability. The thermostability of the enzymes was analyzed by placing enzyme samples in a water bath at 70°C and by assessing the residual enzymatic activity from 0 to 5 h. The tolerance of the lipases to methanol was evaluated by dissolving enzyme samples in a 30% methanol solution and assessing the residual enzymatic activity from 0 to 100 h. Each sample was assayed in triplicate and the average value was determined.

Kinetic parameters. The interfacial kinetic parameters k_{cat} and K_m were determined in a heterogeneous medium using pNPP as the substrate according to the method of Burdette and Quinn (1986).

Circular dichroism (CD) experiments. CD spectra were recorded on a JASCOJ-810 automatic spectropolarimeter (Jasco, Tokyo, Japan). Concentration of the purified proteins was maintained at 100 μ g/ml. All spectral analyses were performed in 10 mM phosphate buffer, pH 7.0, and the scans were carried out between wavelengths 260 and 190 nm.

Results

Design of the new RML lipase with a new propeptide region

Considering the importance of propeptide, we constructed two new kinds of RML by replacing the propeptide

Fig. 2. Scheme of the chimeric lipases.

Fig. 3. SDS-PAGE analysis of recombinant lipases (line 1, wildpro-RML; line 2, RCLpro-RML; line 3, ROLpro-RML).

region with that from either RCL or ROL. The enzymes belong to *Rhizopus* lipases, and amino acid sequence alignment showed that ROL is closely related to RCL (82.16% homology). RML, however, belongs to the *Rhizomucor* genus and therefore shares less homology with RCL and ROL. The sequence identity between RML and RCL is 55.97% and between RML and ROL is 54.85%. The difference between the propeptides is greater than that between the mature polypeptides. The sequence identity between RML-pro and RCL-pro is 26.09% and between RML-pro and ROL-pro is 29.51% (Fig. 1). Replacement of the propeptide region of RML with that from RCL or ROL for the heterologous expression is likely to be a big challenge. The chimeric RMLs were constructed by replacing the propeptide region with that from RCL and ROL, and the resulting recombinant constructs were named RCLpro-RML and ROLpro-RML, respectively (Fig. 2).

Expression and enzymatic activity of the lipases

The three kinds of RML with different propeptide regions were expressed in *P. pastoris*: wildpro-RML, RCLpro-RML, and ROLpro-RML. The molecular weight of the expressed proteins was measured by SDS-PAGE (Fig. 3). In the figure, lane 1 is wildpro-RML, lane 2 is RCLpro-RML, and lane 3 is ROLpro-RML. SDS-PAGE analysis revealed that the molecular masses of purified three kinds of enzyme were about 32 kDa, consistent with the reported molecular mass (Wu et al., 1996).

As shown in Fig. 4, after replacement of the propeptide region, the activity of the RCLpro-RML and ROLpro-RML

Fig. 4. Effect of temperature on activity.

Table 1. K_m and k_{cat} of wild pro-RML, ROLpro-RML and RCLpro-RML.

	wildpro-RML	ROLpro-RML	RCLpro-RML
K_m (mM/L)	0.66	1.13	1.35
k_{cat} (min ⁻¹)	21.25 ± 0.8	50.38 ± 2.3	86.25 ± 3.5
k_{cat}/K_m (min ⁻¹ mM ⁻¹ L)	32.20 ± 1.2	44.58 ± 2.04	63.89 ± 2.6

reached a maximum of 6.2 and 3.6 U/mg, respectively, in the *p*NPP assay; this activity was 3.4-fold and 2.0-fold higher, respectively, than that of the RML derived from the wild-type propeptide.

Kinetic parameters

K_m and k_{cat} of the three RMLs were analyzed and are listed in Table 1. The results showed that k_{cat} of RML was improved in both mutants, but K_m values did not change significantly. Both RCLpro-RML and ROLpro-RML showed considerably greater catalytic efficiency (k_{cat}/K_m) than did wildpro-RML, predominantly owing to the higher k_{cat} , which indicates a higher catalytic rate.

Effects of temperature and pH on the enzymatic activity

Temperature and pH are two important reaction parameters for enzymes, and different enzymes have distinct optimal temperatures and pH levels. The reaction conditions for the three kinds of RML were optimized with *p*-NPP serving as the substrate. As one can see in Figs. 4 and 5, the three kinds of RML have different optimal reaction temperatures and the same optimal pH. Figure 4 shows that the optimal reaction temperature for wildpro-RML is 55°C, for RCLpro-RML 35°C, and for ROLpro-RML 44°C. The optimal reaction pH for all three kinds of RML is 7.0 (Fig. 5).

Effects of temperature and methanol on enzyme stability

In this study, thermostability of lipases was measured by placing enzyme samples in a water bath at 70°C and by assessing the residual enzymatic activity after lengthy incubation. The thermostability of the three RMLs was different, wildpro-RML is most tolerant of high tempera-

Fig. 5. Effect of pH on activity.

Fig. 7. Stability in 30% methanol.

Fig. 6. Thermostability of the enzymes.

Fig. 8. The CD spectrum of the three RMLs.

tures, and RCLpro-RML is the most sensitive (Fig. 6).

The tolerance of lipases to methanol was evaluated by dissolving the enzymes in a 30% methanol solution and by assessing the residual enzymatic activity. The results showed that wildpro-RML could retain up to 80% of its activity after 100 h; under the same conditions, the residual enzymatic activity of RCLpro-RML and ROLpro-RML was ~50% (Fig. 7). The above findings indicate that the stability of RML decreased after the changes in the propeptide region.

The CD spectra

After purification and analysis by SDS-PAGE (Fig. 3), the secondary structures of the three types of lipase were studied by CD analysis (Fig. 8). These results showed that the secondary structures of the two mutants were different from that of wildpro-RML.

Discussion

The activity of mutants is higher than that of the wild-RML (Fig. 4). The increase in enzymatic activity in the mutants could be explained by the analysis of kinetic parameters. The kinetic parameters (Table 1) show that k_{cat}

of RML was improved in both mutants, and K_m values did not change significantly after the propeptide region was replaced by the ones from RCL and RML. Both RCLpro-RML and ROLpro-RML showed a considerably greater catalytic efficiency (k_{cat}/K_m) than did wildpro-RML, predominantly owing to the higher k_{cat} .

Thermostability is an important characteristic of an enzyme, and every enzyme has a distinct thermostability. The thermostability is expected to change if the stereostructure of an enzyme is altered. In this study, Wildpro-RML showed better stability than the mutants, either at high temperature or in 30% methanol. At the same time, the optimal reaction temperature also decreased. It is likely that the stereostructure of RML changed after its propeptide region was substituted. The change can also be seen in the CD spectra of the three RMLs (Fig. 8).

Propeptide regions often function as IMCs which facilitate protein folding (Shinde and Inouye, 2000). Shinde et al. (1997) found that the same mature polypeptide can fold into an altered conformation because of the changed IMC. In the present study, we did not mutate but substituted the propeptide region with a propeptide region from other enzymes. The results show that the two new kinds of RML with new propeptide regions can be successfully expressed

in *P. pastoris*. The changes in the RML propeptide caused the same mature polypeptide to fold into different conformations, each with distinct characters. That means that the proteins in lanes 1, 2, and 3 may have different stereostructures, even though they have the same molecular weight and polypeptide sequence (Fig. 3).

Considering the increased enzymatic activity with the decrease of stability after the replacement of the propeptide region, we can speculate that it is possible to synthesize new propeptides by combining parts of the original prosequence and of new propeptides. The resulting synthetic propeptides may not only have an enhanced enzymatic activity but may also keep the stability of a wild-type enzyme. This idea may be very useful for the optimization of protein properties if it can be implemented.

Conclusions

From these results, we can draw some conclusions. First, properties of some enzymes can be altered by changing the propeptide region. Second, the replacement of the propeptide region is possible among enzymes from different genera (without changes in the mature peptide), and some properties of the enzyme can be improved by this strategy. Finally, it is possible to manipulate the stereochemical structure and characteristics of an enzyme via the synthesis of a new propeptide region.

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