Appl Microbiol Biotechnol (2001) 55:454–462
DOI 10.1007/s002530000537

ORIGINAL PAPER

S. Takahashi · M. Ueda · A. Tanaka

Function of the prosequence for in vivo folding and secretion of active *Rhizopus oryzae* lipase in *Saccharomyces cerevisiae*

Received: 23 May 2000 / Received revision: 11 August 2000 / Accepted: 1 September 2000 / Published online: 13 March 2001

Abstract The role of the prosequence of *Rhizopus oryzae* lipase (ROL) with a preprosequence was analyzed by an expression system using *Saccharomyces cerevisiae*. When the mature portion of ROL (mROL) fused to the pre-α-factor leader sequence was expressed, secretion of active mROL was not observed. However, when mROL was synthesized together with the prosequence in *trans* (individually and coincidentally), secretion of active mROL was observed. The results indicate that the prosequence of ROL helped correct folding of mROL and its subsequent secretion from the yeast cells, and that physical linkage (*cis*) of the prosequence to the mature region was not prerequisite. From the expression of the ROL mutants with deletions at the N-terminal end of the prosequence together with mROL in *trans*, the residues from 20 to 37 in the prosequence were essential for the secretion, and those from 38 to 57 were essential for the formation of the active ROL and might play a role as an intramolecular chaperone. The results using the fragment of the prosequence confirmed that these residues (20–57) were significant for in vivo folding and secretion of active mROL.

Introduction

Secreted proteins of prokaryotes and eukaryotes commonly have precursor forms, which are subsequently processed into mature proteins by the action of one or more hydrolases. Presequences at the N-terminal region of the precursor proteins function as the signal peptide for secretion across the membrane (Randall and Hardy 1989). In addition to the presequence, many proteins such as hydrolases, growth factors, and hormones possess additional polypeptides, called prosequences, at the N-terminus, C-terminus, or both termini of the precursor proteins.

It is known that the prosequences in some proteolytic enzyme precursors inhibit the activity of the mature portions (Neurath 1989). However, some of the prosequences seem to have other functions. The prosequences of several serine proteases, such as subtilisin E of *Bacillus subtilis* (Zhu et al. 1987), α-lytic protease of *Lysobacter enzymogenes* (Silen and Agard 1989), and carboxypeptidase Y of *Saccharomyces cerevisiae* (Winther et al. 1994), are shown to have the function to help folding of the mature portions, and covalent linkage between the prosequences and the mature portions is not necessary for the prosequence-assisted folding. In addition to proteases, proteins such as growth factors (Gray and Mason 1990), neuropeptides (Suter et al. 1992), hormones (Clark and Steiner 1969), and plasma proteins (Voorberg et al. 1993) also require their prosequences for the correct folding. The prosequence-mediated folding mechanism thus seems to be a widespread phenomenon. The folding mechanism is clearly different from that of proteins assisted by molecular chaperones. As these prosequences are covalently attached to the mature proteins to be folded, the prosequences are called "intramolecular chaperones (IMC)" to distinguish them from the molecular chaperones (Inouye 1991). In comparison with proteases, little is known about the functional role of the prosequences of lipases.

Lipase secreted from *Rhizopus oryzae* (ROL) is synthesized as a precursor form with a presequence (23 amino acid residues) and a prosequence (97 amino acid residues) at the N-terminal side of the mature portion (268 amino acid residues) (Beer et al. 1998), similar to lipases of *R. delemar* (Haas et al. 1991), *R. niveus* (Kohno et al. 1994), *Rhizomucor miehei* (Boel et al. 1988), and *Fusarium heterosporum* (Nagao et al. 1994).

ROL was expressed in *Escherichia coli* as an insoluble form (Haas et al. 1991; Beer et al. 1996). In *E. coli*, the prosequence of ROL might play a similar role to the prosequence of bovine pancreatic trypsin inhibitor

S. Takahashi · M. Ueda · A. Tanaka (✉)
Laboratory of Applied Biological Chemistry,
Department of Synthetic Chemistry and Biological Chemistry,
Graduate School of Engineering, Kyoto University, Yoshida,
Sakyo-ku, Kyoto 606-8501, Japan
e-mail: atsuo@sbchem.kyoto-u.ac.jp
Tel.: +81-75-7535524, Fax: +81-75-7535534

(BPTI) in refolding these mature portions. These prosequences seem to facilitate the folding by providing an intramolecular thiol-disulfide reagent (Weissman and Kim 1992). However, the function in eukaryotic cells and essential portions in the prosequence are not clear yet.

The active ROL has been successfully secreted extracellularly with high efficiency from *S. cerevisiae* when the pro-form (ProROL)-encoding gene fused to the pre-α-factor-encoding gene was expressed (Takahashi et al. 1998). However, when the mature ROL (mROL)-encoding gene directly fused to the pre-α-factor-encoding gene was expressed in *S. cerevisiae*, the activity was not detected in the culture supernatant and in the cells. Therefore, it was assumed that the prosequence of ROL might support the folding and/or the secretion of mROL in vivo.

Several *Pseudomonas* lipases require other proteins for folding and secretion, called foldase, modulator proteins, or activator proteins (Hobson et al. 1993; Frenken et al. 1993; Iizumi and Fukase 1994; Ihara et al. 1995). Extracellular lipase of *P. cepacia* DSMS3959 encoded by *lipA* required a product of the second gene, *limA*, which acts in *trans*, for its activation in either *E. coli* or *B. subtilis* as a host (Jørgensen et al. 1991; Hobson et al. 1993). Furthermore, in vitro refolding experiments showed that the lipase activity could be recovered in the presence of the LimA protein. Since no covalent binding between the lipase and LimA protein was demonstrated after renaturation, the LimA protein apparently had a role as a molecular chaperone (Hobson et al. 1993).

The present study deals with the function of the prosequence of ROL for the formation of the active lipase by using the expression system in *S. cerevisiae*. The prosequence was found to be essential for the secretion and the formation of mROL, and covalent linkage between the prosequence and mROL was not necessary. We therefore propose that the prosequence of ROL leads to correct folding of ROL as an intramolecular chaperone to pass through the secretory pathway. Moreover, the essential regions in the prosequence for the secretion and the formation of active mROL were determined.

Materials and methods

Strains, media, and growth conditions

Escherichia coli strain DH5α[*F*$^-$, *endA1*, *hsdR17*(r_k^-, m_k^+), *supE44*, *thi-1*, *λ*$^-$, *recA1*, *gyrA96*, *ϕ80dlacZΔM15*] was used as a host for recombinant DNA manipulation. *Saccharomyces cerevisiae* KDH3 (*MATa*, *kex2::HIS3*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) (Takahashi et al. 1999) was used as the host for the protein production. *E. coli* was grown in LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast-extract, 0.5% (w/v) sodium chloride] containing 0.1% (w/v) glucose and 50 μg/ml ampicillin. Yeast was routinely precultivated in 10 ml SD medium that contained 0.67% (w/v) yeast nitrogen base without amino acid (Difco, Detroit, Mich., USA) and 2% (w/v) glucose supplemented with 0.003% L-leucine and 0.002% L-adenine in an 18-mm-diameter test tube at 30 °C for 24 h and cultivated aerobically in 100 ml SDC medium [SD medium containing 2% (w/v) casamino acids (Difco)] in a 500-ml shaking flask at 30 °C for 48 h.

Transformation of *S. cerevisiae*

Transformation of *S. cerevisiae* cells was carried out by the lithium acetate method (Ito et al. 1983) using the Yeastmaker Yeast Transformation System (Clontech Labo., Palo Alto, Calif., USA). The transformants were isolated by incubation at 30 °C for 48 h on a plate of SD medium.

Construction of plasmids

For the expression of the genes encoding the pre-α-factor fused proteins to the mature portion of ROL (mROL), to mROL with the prosequence (ProROL), and to mROL with the pro-α-factor leader sequence (PαROL), a plasmid (pWGP3) introducing the blunted 1.3-kbp *Hin*dIII fragment containing glyceraldehyde-3-dehydrogenase (GAPDH) promoter and terminator from pYE22 m (Sawai-Hatanaka et al. 1995) into the *Pvu*II site of pMW1 (Kanai et al. 1996) was constructed. The genes encoding PαROL, ProROL, and mROL fused to the pre-α-factor leader sequence were obtained from pWRL1, pWRL2, and pWRL3 (Takahashi et al. 1998), respectively, by digestion with *Bgl*II and *Xho*I, and ligated with pWGP3 at the *Bam*HI and *Sal*I sites. The expression plasmids for PαROL, ProROL, and mROL were named pWGRL1, pWGRL2, and pWGRL3, respectively.

For the expression of the genes encoding the fusion protein of the prosequence of ROL with the pre-α-factor and the prepro-α-factor leader sequence, a plasmid, pUGP3, was first constructed by cloning the GAPDH promoter and terminator into the *Bam*HI and *Pvu*II of pMT34(+3) (Tajima et al. 1985). The genes encoding the fusion protein of the prosequence of ROL with pre-α-factor (Pro) and the prepro-α-factor leader sequence (Pα), which were introduced by the termination codon just after the genes, were amplified by the polymerase chain reaction (PCR) using the primers I3SU (5′-CTATAGATCTGTCGACATGAGATTTCCTTC-3′) and ProXh (5′-TTACCCTCGAGTTAGGCGCTGTTGGTAGATCCAGAGAGGC-3′) with pWRL2 as the template for the prosequence, and using the primers I3SU and MFa3p (5′-AAGCCTCGAGTTATCTTTTATCCAAAGATACCCCTTC-3′) with pUC19α (Takahashi et al. 1998) as the template for the prepro-α-factor leader sequence. The amplified fragments were digested by *Bgl*II (underlined) and *Xho*I (underlined) and then ligated with pUGP3 at the *Bam*HI and *Sal*I sites. The expression plasmids for the prosequence and the prepro-α-factor leader sequence were named pUGPro and pUGMA, respectively.

Various deletion mutants at the N-terminus in the prosequence of ProROL were constructed by PCR with pWRL2 as the template. The primers used were dProp1 (5′-TAAATCTAGATCTTCCATCACCGCCGTCTCTGCATCTGAC-3′), dProp2 (5′-ATCTTCTAGATCTGCCCTCCCTCCTCTCATTTCCAGCCGTTG-3′), dProp3 (5′-TAACTCTAGAAGTAAAAGCGATCTTCAAGCTGAACCTTAC-3′), dProp4 (5′-ATGGTCTAGATCCCATGGTGGCAACCTGACATCCATCGG-3′), dProp5 (5′-CGGATCTAGAGATGACAATTTGGTTGGTGGCATGACTTTGG-3′), dProp6 (5′-GGATTCTAGAAGAGATGCTCCTCCTATCAGCCTCTCTGG-3′), and I3XR (5′-CCTTGGTTTCCTCGAGTTTTACAAACAGCTTCC-3′). The amplified fragments were digested by *Xba*I (underlined) and *Xho*I (underlined), and then ligated with pUC19pα (Takahashi et al. 1998) containing the pre-α-factor leader sequence gene. The *Xba*I site between the pre-α-factor leader sequence-encoding gene and the *mROL* gene was converted to the original sequence by site-directed mutagenesis (Ito et al. 1991) using the primers MdPro1 (5′-GCAGCATCCTCCGCATTAGCTTCTTCCATCACCGCCGTCTC-3′), MdPro2 (5′-GCAGCATCCTCCGCATTAGCTTCTGCCCTCCCTCCTCTCA-3′), MdPro3 (5′-GCAGCATCCTCCGCATTAGCTAGTAAAAGCGATCTTCAAGC-3′), MdPro4 (5′-GCAGCATCCTCCGCATTAGCTTCCCATGGTGGCAACCTGAC-3′), MdPro5 (5′-GCAGCATCCTCCGCATTAGCTGATGACAATTTGGTGGTGGC-3′), and MdPro6 (5′-GCAGCATCCTCCGCATTAGCTAGCGATGCTCCTCCTATCAGCC-3′) (mutated sequences underlined). The fragments were digested by *Sal*I and *Xho*I and ligated with pWI3

(Kanai et al. 1996). The deletion fragments ligated with pWI3 were digested by *Bgl*II and *Xho*I, and the *Bgl*II/*Xho*I fragments were ligated with pWGP3 at the *Bam*HI and the *Sal*I sites. The plasmids for the expression of the deletion mutants of the prosequence in ProROL were named pWdPL1, pWdPL2, pWdPL3, pWdPL4, pWdPL5, and pWdPL6, respectively. For expression of the deletion mutants of the prosequence, the deleted prosequence-encoding genes fused to the pre-α-factor leader sequence-encoding gene introduced a termination codon just after the genes were amplified from pWdPL1-6 by PCR using the primers I3SU and ProXho (5′-TTACCCTCGAGTTAGGCGCTG-TTGGTAGATCCAGAGAGGC-3′). The amplified fragments were digested by *Bgl*II and *Xho*I (underlined) and ligated with pUGP3 at the *Bam*HI and *Sal*I sites. The plasmids for the expression of the deletion mutants of the prosequence were named pUdP1, pUdP2, pUdP3, pUdP4, pUdP5, and pUdP6, respectively.

For the expression of the fragments of the prosequence, the plasmid pUGPS was constructed by insertion of the gene encoding the pre-α-factor leader sequence by annealing of the synthetic oligonucleotides containing the *Bam*HI site and termination codon [Ap1 (5′-GATCATGAGATTTCCTTCAATTTTTACTGCAGTTT-TATTCGCAGCATCCTCCGCATTAGCTGCTGGATCCTAA-3′) and Ap2 (5′-TCGATTAGGATCCAGCAGCTAATGCGGAGGAT-GCTGCGAATAAAACTGCAGTAAAAATTGAAGGAAATCTC AT-3′)] into the *Bam*HI site of pUGP3. The genes encoding the fragments of the prosequence were amplified by PCR with pWRL2 as a template. The primers used were P1 (5′-ATGC-AGATCTGTTCCTGTTTCTGGTAAATCTGGATCT-3′) and P2 (5′-ATGCAGATCTGGCGCTGTTGGTAGATCCAGAGAGGC-3′) for the whole prosequence (residues 1–97), P3 (5′-ATGCAGA-TCTGCCCT-CCCTCCTCTCATTTCCAGCCG-3′) and P4 (5′-ATGCAGATCTCTCATACCATT-CTGTATTCTTTTGCATGT-3′) for residues 20–57. The amplified genes were digested by the *Bgl*II (underlined) and ligated with pUGPS at the *Bam*HI site. Accurate constructions of all plasmids were confirmed by nucleotide sequencing.

Recovery of secreted proteins

The culture supernatants of transformants were obtained by centrifugation at 4,000 *g* for 10 min at 4 °C, and then one-tenth volume of 100% trichloroacetic acid (TCA) containing 4 mg/ml deoxycholic acid was added to them. After the mixtures were left for 15 min on ice, insoluble materials were collected by centrifugation at 12,000 *g* for 20 min at 4 °C. The pellets were washed with 100% acetone (–20°C) and dried in vacuo. The dried pellets were dissolved and subjected to SDS-PAGE.

Preparation of cell-free extracts

Yeast cells (OD_{600}: 20) were harvested by centrifugation at 3,000 *g* for 5 min. Cells were washed three times with 1 ml ice-cold water each time and resuspended in 0.5 ml ice-cold disruption buffer (1 mM PMSF, 5 mM EDTA, 3 µg/ml leupeptin, 3 µg/ml pepstatinA in 50 mM Tris-HCl, pH 7.5). Extracts were prepared by vortexing the cells with glass beads (0.45–0.5 mm diameter) for 30 s 15 times and centrifuging at 14,000 *g* for 20 min. The supernatants obtained were used as cell-free extracts. All operations were performed at 0–4 °C.

Enzyme assay

Lipase activity was measured using Lipase Kit S (Dainippon Pharmaceutical Co., Osaka, Japan) according to the application protocol recommended by the supplier. One unit of enzyme activity was defined as the amount of 1 µmol 2,3-dimercaptopropan-1-ol formed from 2,3-dimercaptopropan-1-ol tributyl ester per minute (Kurooka et al. 1977).

Electrophoresis and Western blot analysis

SDS-PAGE (12.5% (w/v) polyacrylamide gel) was performed by the method of Laemmli (1970). Tricine-SDS-PAGE [10% (w/v) polyacrylamide gel] was performed by the method of Schagger and von Jagow (1987). In Western blot analysis, after SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass., USA) using a semidry blotting apparatus (ATTO, Tokyo, Japan). In the dot blot analysis, 0.5 ml of the culture supernatants was mixed with an equal volume of the dot blot buffer [100 mM Tris-HCl (pH 6.8), 6% (w/v) sodium dodecyl sulfate (SDS), 80 mM 2-mercaptoethanol] and boiled for 5 min, and then 0.5 ml of the sample solution was blotted on the PVDF membrane. The ROL on the membrane was immunoblotted with a polyclonal antiserum for ROL prepared by injection of purified rProROL produced by *S. cerevisiae* to rabbits, and the relative amount of ROL was determined by NIH Image 1.62. Before use, the antiserum was diluted 1,000-fold with PBS containing 0.5% (v/v) Tween 20 and pretreated with yeast cells for 1 h at room temperature to remove the nonspecific adsorption to ROL. Western blot analysis was conducted as described previously (Ueda et al. 1989).

Analysis of N-terminal amino acid sequence

After SDS-PAGE, the proteins were transferred onto a sheet of ProBlott (Applied Biosystems, Foster City, Calif., USA). The filter was placed in a protein sequencer 492 (Applied Biosystems) and analyzed as recommended by the manufacturer.

Results

Trans-expression of the prosequence and the mature portion

When the mature region of ROL (mROL) fused to the pre-α-factor leader sequence was expressed in *S. cerevisiae*, lipase activity was not detected in either the culture supernatant or the cell homogenate, although such activity was detected in both fractions when mROL having the prosequence (ProROL) was expressed ("*cis*-expression") (Takahashi et al. 1998). Therefore, the prosequence might support the correct folding of its mature portion. In the function of the prosequence of the subtilisin E from *B. subtilis* (Shinde and Inouye 1993) on its correct folding, covalent linkage between its mature portion and the prosequence was not necessary (Zhu et al. 1987). To examine whether or not covalent linkage between the prosequence of ROL and mROL is essential for the formation of the active ROL, the prosequence and mROL (Fig. 1) were coexpressed in *trans* (individually and coincidentally) in *S. cerevisiae* KDH3, a *kex2* disruption strain (Takahashi et al. 1999). After the transformant cells were cultured in the SDC liquid medium for 48 h at 30 °C, the lipase activity in the culture supernatants and the cell-free extracts was determined (Fig. 2). The activity was low in both the culture supernatant (1.48 U/l) and the cell-free extract (16.3 U/l) when only mROL was expressed (Fig. 2, A, B, lane 3), whereas significant activity was detected in both fractions when mROL was coexpressed in *trans* with Pro (Fig. 2, A, B, lane 2). The activity in the culture supernatant and the cell-free extract

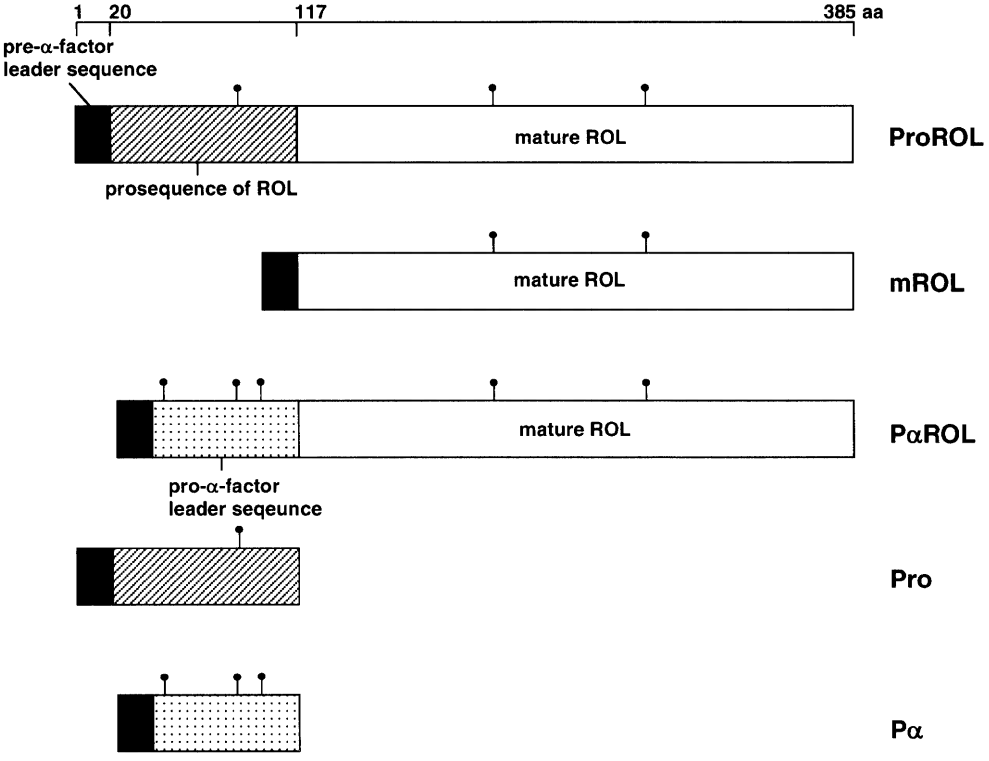

Fig. 1 Schematic representation of the protein products. The structures of the recombinant proteins used in this study are shown (named as described at right side). The pre-α-factor leader sequence (*filled box*), the prosequence of ROL (*hatched box*), mature portion of ROL (mROL) (*open box*), and the pro-α-factor leader sequence (*stippled box*). *Closed circles* above the diagrams indicate the potential N-glycosylation sites

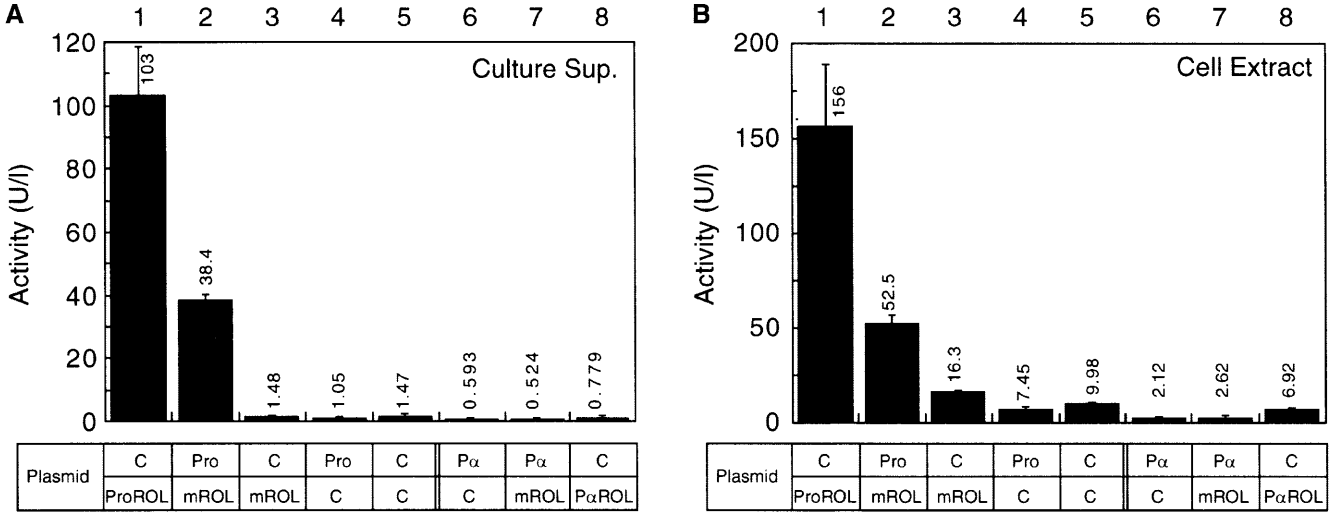

Fig. 2A, B Extra- (**A**) and intracellular (**B**) activity in the *trans*-expression of mROL and the prosequences. The culture supernatants, *Culture Sup.*, and the cell-free extracts, *Cell Extract*, were prepared after cultivation for 48 h and the activity was determined as described in the Materials and methods section. *Plasmid* indicates the expression plasmids transformed for each gene encoding the recombinant protein described in Fig. 1. *C* indicates the control plasmid. The data shown are mean±SE, *n*=3

was 38.4 U/l (Fig. 2A, lane 2) and 52.5 U/l (Fig. 2B, lane 2), respectively. These results showed that covalent linkage between the prosequence and mROL was not essential for the formation and secretion of the active ROL. When ProROL (Fig. 1) was expressed, the activity in the culture supernatant and the cell-free extract was 103 U/l (Fig. 2A, lane 1) and 156 U/l (Fig. 2B, lane 1), respectively, suggesting superiority of *cis*-expression over *trans*-expression.

To determine the specificity of the prosequence for the activation of mROL, mROL was coexpressed in *cis* (PαROL) and *trans* (Pα and mROL) with the pro-α-factor leader sequence (Fig. 1). No significant activity was detected in either the culture supernatants (Fig. 2A, lanes 7 and 8) or the cell-free extracts (Fig. 2B, lanes 7 and 8), irrespective of *trans*- and *cis*-expression. These results suggested that the prosequence of ROL is specifically required for the formation of active ROL.

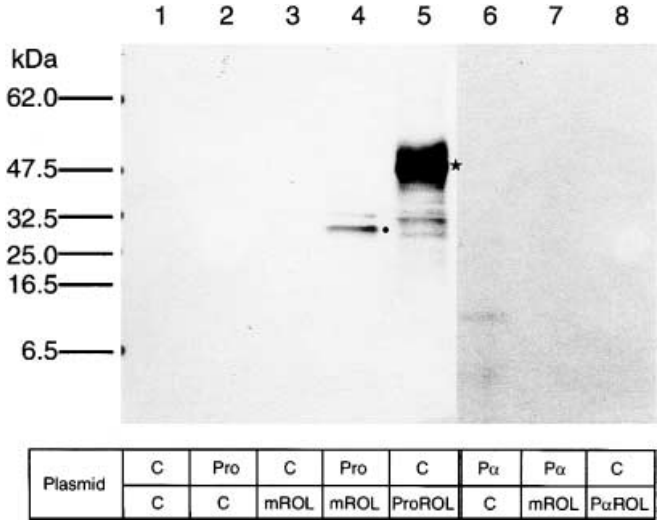

Fig. 3 Identification of extracellular ROL. Proteins were obtained by TCA precipitation from 1 ml of the culture supernatants after cultivation for 48 h and separated by 10% (w/v) tricine-SDS-PAGE, and then ROL was detected by Western blotting using a polyclonal ProROL-specific rabbit antiserum as described in the Materials and methods section. *Plasmid* indicates the expression plasmids transformed for each gene encoding the recombinant protein described in Fig. 1. *C* indicates the control plasmid. Molecular mass markers are shown on the *left*. The *asterisk* and the *dot* indicate ProROL and mROL, respectively

Secretion of the mature portion in the *trans*-expression with the prosequence

To confirm the extracellular production of mROL, Western blot analysis was carried out. A band corresponding to mROL was observed at about 30 kDa (Fig. 3, lane 4), which corresponded to the same as the molecular mass deduced from the nucleotide sequence of mROL, in the culture supernatant of the cells coexpressing mROL with Pro in *trans*. The N-terminal amino acid sequence of mROL was confirmed to be SDGGKVVAAT, which was identical to the deduced amino acid sequence. Such a band was not observed with the culture supernatant of the cells expressing only mROL or coexpressing with Pα in *trans* (Fig. 3, lanes 3 and 7). The band corresponding to the prosequence, about 10 kDa deduced from the nucleotide sequence, was not observed in the culture supernatants of the cells expressing only Pro or coexpressing Pro with mROL in *trans* (Fig. 3, lanes 2 and 4). The band corresponding to ProROL was observed at about 48 kDa with the culture supernatant of the cells expressing ProROL (Fig. 3, lane 5), whereas the fusion protein of mROL and the pro-α-factor was not detected (Fig. 3, lane 8). These results indicate that the prosequence could specifically support the secretion and formation of active ROL in *cis*- and *trans*-expression systems in vivo.

Effect of the amino-terminal deletion of the prosequence on activity of mROL in the *trans*-expression

To clarify the functional region of the prosequence on the secretion and the formation of active ROL, the N-terminus of the prosequence was truncated (Fig. 4). The genes encoding the deleted prosequences fused to the pre-α-factor leader sequence were coexpressed in *trans* with mROL in *S. cerevisiae* KDH3. The transformant cells were cultivated in SDC liquid medium for 48 h at 30 °C. Sufficient activity was still observed in the culture supernatant of the mutants with the prosequences deleted 8 (P89) and 19 residues (P78) from the N-terminus (Fig. 5A, lanes 3 and 4). The activity was decreased from 52.1 U/l to 18.8 U/l by the deletion of 8 residues (Fig. 5A, lanes 2 and 3), and recovered to 42.4 U/l by the deletion of 19 residues (Fig. 5A, lanes 3 and 4), whereas the activity was markedly reduced to 1.50 U/l by deletion of more than 37 residues (P60-mROL) (Fig. 5A, lanes 5–9). However, sufficient activity, 88.0 U/l, was observed in the cell-free extract in P60 (Fig. 5B, lane 5). The activity in the cell-free extract with P60 was much higher than that observed in Pro, 66.8 U/l (Fig. 5B, lane 2). Deletion of more than 57 residues (P40-mROL) (Fig. 5B, lanes 6–9) markedly reduced the activity even in the cells. These results indicated that the region from 38 (P60) to 57 (P40) residues was necessary for the formation of the active ROL.

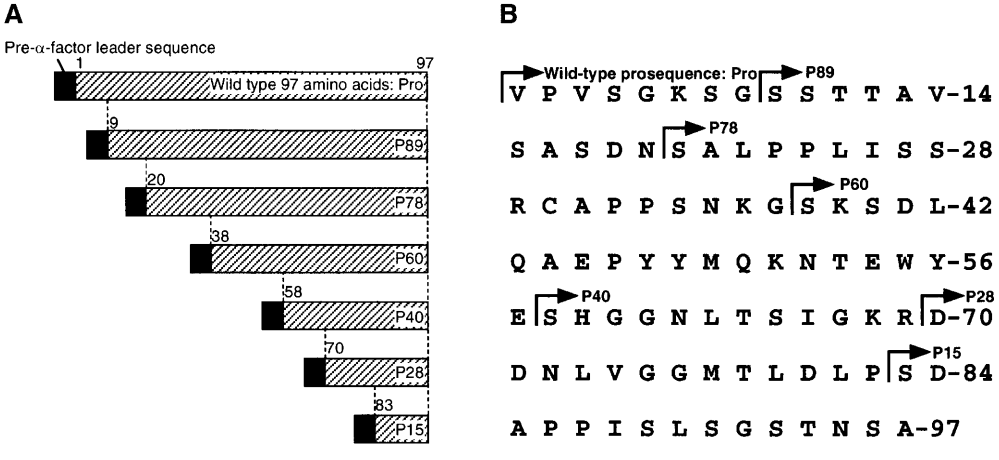

Fig. 4A, B Schematic representation (**A**) and amino acid sequences (**B**) of the N-terminal deletions of the prosequence of ROL. **A** Structures of the deleted prosequence of ROL fused to the pre-α-factor leader sequence (named as described at the *right* side in the box). Numbers above the diagrams correspond to the amino acid residues from the N-terminus of the prosequence. **B** Amino acid sequence of the prosequence of ROL (Takahashi et al. 1998). *Arrows* show the initial amino acid residues of each deletion of the prosequence

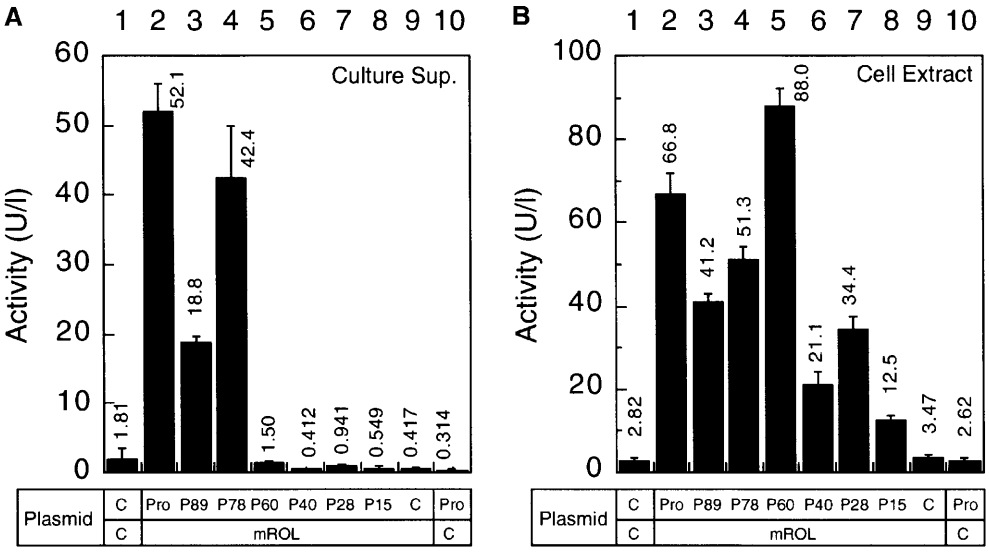

Fig. 5A, B Effect of the N-terminal deletion in the prosequence of ROL on the extra- (**A**) and intracellular (**B**) activity in the *trans*-expression with mROL. The culture supernatant, *Culture Sup.*, and the cell-free extract, *Cell Extract*, were prepared after cultivation for 48 h and the activity was determined as described in the Materials and methods section. *Plasmid* indicates the expression plasmids transformed for each gene encoding the recombinant proteins described in Figs. 1 and 4. *C* indicates the control plasmid. The data shown are mean±SE, *n*=3

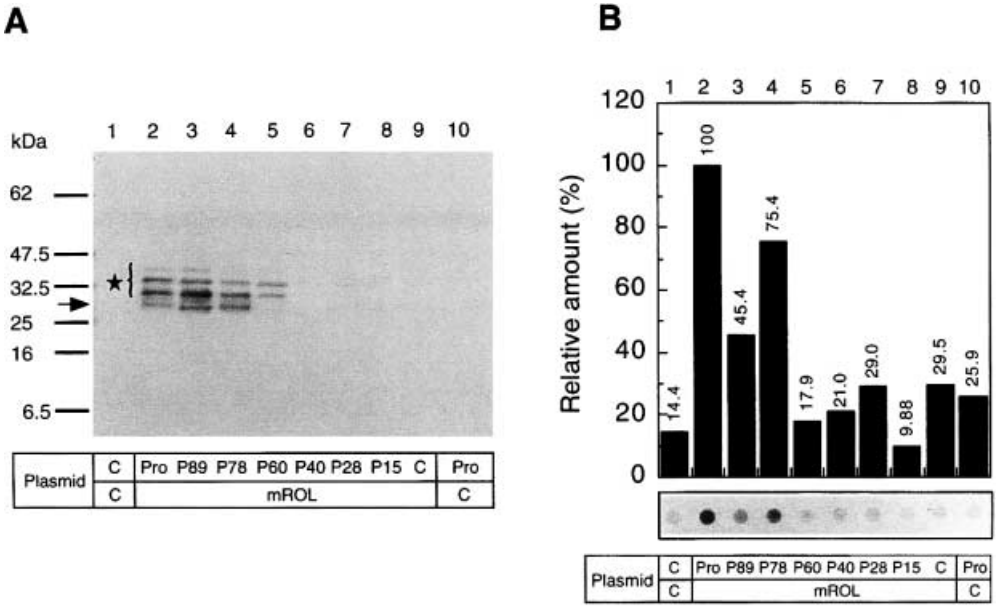

Fig. 6A, B Effect of N-terminal deletion in the prosequence on the secretion of mROL in the trans-expression. **A** Proteins were obtained by TCA precipitation from 2 ml of the culture supernatants after cultivation for 48 h and separated by 12.5% (w/v) SDS-PAGE, and then detected by Western blotting using the polyclonal ProROL-specific rabbit antiserum as described in the Materials and methods section. The *arrow* indicates the band corresponding to mROL. Molecular mass markers are shown on the *left*. The *asterisk* indicates the N-glycosylation forms of mROL. **B** Relative amounts of secreted mROL. Culture supernatants used in **A** were blotted on the membrane as described in the Materials and methods section, and ROL was detected by Western blotting using the polyclonal ProROL-specific rabbit antiserum, and then analyzed by NIH Image 1.62. The highest value was represented as 100%. *Plasmid* indicates the expression plasmids transformed for each gene encoding the recombinant proteins described in Figs. 1 and 4. *C* indicates the control plasmid

Secretion of the mature portion in the *trans*-expression with various prosequences

Western blot analysis for ROL revealed that mROL (about 30 kDa) was present in the culture supernatants of the cells coexpressing in *trans* with Pro, P89, or P78 (Fig. 6A, lanes 2–4). Additional bands above 30 kDa, observed in the coexpression system with Pro, P89, P79, and P60 (Fig. 6A, lanes 2–5), might be the N-linked glycosylated forms, because these bands disappeared with Endo-H treatment, the 30 kDa band remaining (date not shown). From these results, it can be concluded that the region from residues 20–37 of the prosequence is essential for secretion of the mature portion, and that residues 38–57 are necessary for correct folding to form the active ROL.

To make clear the relationship between the activity and the amount of mROL in the culture supernatant of

cells coexpressing mROL and the deleted prosequences in *trans*, we carried out dot blot analysis for the secreted ROL (Fig. 6B). The total amount of ROL secreted correlated well with the level of activity (Figs. 5A, 6B). The results indicated that the activity detected in the culture supernatant was dependent on the amount of the secreted ROL, and did not result from the change of activity of the secreted ROL.

To analyze more exactly the functional region of the prosequence determined here, the genes encoding the fragments of the prosequence fused to the pre-α-factor were coexpressed in *trans* with the gene encoding mROL in *S. cerevisiae* KDH3. The transformant cells were cultivated in SDC liquid medium for 48 h at 30 °C. In the *trans*-expression of mROL and the various fragments of the prosequence, sufficient activity was observed only in the *trans*-expression with a fragment from residues 20–57 of the prosequence. Surprisingly, in the *trans*-expression of mROL with fragments from residues 20–37 and 38–57, individually, no activity was detected in any fractions, although the reason for this remains unknown. The activity in the supernatant (14.6 U/l) was one-fifth of that with the wild-type prosequence (62.5 U/l), and in the cell-free extract much higher activity (191 U/l) was observed than with the wild-type prosequence (121 U/l). Moreover, Western blotting of the culture supernatants showed that mROL was detected at about 30 kDa in the *trans*-expression of mROL with the fragment from residues 20–57, in addition to the wild-type prosequence (data not shown). These results suggest that the region from residues 20–57 is significant for in vivo folding and secretion of active mROL, and that in that region residues 38–57 may play a role as an intramolecular chaperone.

Discussion

We analyzed the possible function of the prosequence of *R. oryzae* lipase (ROL) as an intramolecular chaperone in the secretion and formation of its mature portion in the expression system using *S. cerevisiae* as a host. The fact that the ROL is functionally produced only when expressed as the pro-form in *S. cerevisiae* (Takahashi et al. 1998) indicated that the expression system in *S. cerevisiae* would be adequate to evaluate the function of the prosequence for the formation of the active ROL in vivo. In addition, as ROL is originally secreted from the filamentous fungi, *S. cerevisiae* is better than *E. coli* because eukaryote and prokaryote have different secretion pathways.

Secretion and formation of the active ROL disappeared with complete deletion of the prosequence (Fig. 2). However, the prosequence synthesized in *trans* was effective for the recovery of secretion and formation of mROL in vivo (Fig. 2). These results indicate that the prosequence acts as an intramolecular chaperone that organizes correct folding of the mature portion of ROL and probably works after translocation of the ROL across the ER membrane into the lumen. In general, secretory proteins that are not folded correctly are retained in the ER lumen (Lippincott-Schwartz et al. 1988). Therefore, it is presumed that mROL folded incorrectly is retained in the ER lumen and degraded. However, the folding of mROL in the *trans*-expression was successfully performed similarly to that in the *cis*-expression. As the result, correct folding of the mROL in *trans* could be performed.

The ROL has three potential sites of N-glycosylation (N-X-Ser/Thr, where X can be any amino acid except for proline) (Tanner and Lehle 1987), one being in the prosequence and others in the mature portion (Fig. 1). In the *cis*-expression of the prosequence and mROL, N-glycosylation was observed only in the prosequence but not in the mature portion (Takahashi et al. 1999), whereas, in the *trans*-expression, glycosylation occurred in the mature portion (Fig. 6A). Since glycosylation is dependent on the conformation of a protein (Tanner and Lehle 1987), it was suggested that the folding rate of mROL coexpressed in *trans* with the prosequence might be slower than that in the *cis*-expression.

The prosequence has also been observed to play a role as an intramolecular chaperon in several proteases, such as subtilisin (Zhu et al. 1987), α-lytic protease (Silen and Agard 1989), aqualysin (Lee et al. 1992), and carboxypeptidase Y (Winther et al. 1994). The prosequences in these proteases, however, have no significant sequence similarity to known molecular chaperones. It is reported that many charged amino acids are presented in the prosequences of such proteases and play an important role in the folding (Winther and Sørensen 1991). The prosequence of ROL has no significant similarity to the sequences of proteases and other molecular chaperones. Additionally, the prosequence does not have many charged amino acids: 17.5% (6Asp, 3Glu, 5Lys, 1His, and 2Arg; net charge, +1) compared to 19.7% in the mature portion (15Asp, 9Glu, 15Lys, 7His, and 9Arg; net charge, +9). It is quite interesting that the prosequences of lipase and protease have a similar function on folding, because they are functionally, structurally, and also evolutionarily different enzymes except that the lipase has catalytic amino acids (Ser-His-Asp), like serine proteases such as chymotrypsin and subtilisin.

Despite the putative functional role of the prosequences of proteases in folding, very little is known about the specific regions important for this function. Kobayashi and Inouye (1992) have identified three hydrophobic regions within the subtilisin E prosequence from *B. subtilis*, which plays an important role in the folding of prosubtilisin, presumably by interaction of these regions with a portion of the mature enzyme.

In the case of ROL produced in *E. coli* and refolded, Beer et al. presumed that cysteine-30 (Fig. 4B) in the prosequence might play a key role in facilitating the folding of the enzyme and work as an intramolecular-disulfide reagent (Beer et al. 1996), analogously to the role of the cysteine residue in the prosequence of pro-bovine pancreatic trypsin inhibitor (BPTI) previously suggested (Weissman and Kim 1992). Although the secre-

tion of the active mROL was greatly reduced by the deletion of the residues containing cysteine-30 (Fig. 5A, lane 5), activity was still observed in the cell (Fig. 5B, lane 5). In addition, the mutation from cysteine-30 to serine (data not shown) did not affect the activity in either extra- (39.6 U/l) or intracellular (42.7 U/l) fractions. On the other hand, the activity in the cell was greatly reduced by the deletion of residues 38–57 in the prosequence (Fig. 5B, lane 6), suggesting that residues 38–57 are essential for the formation of the active ROL in vivo, although only the fragment from residue 20 to residue 57 was functional in the coexpression of mROL with the fragment of the prosequence. The secondary-structure analysis using the method of Chou and Fasman (1978) predicted that the region from residues 38–57 constitutes one α-helical structure. This is a common structural feature of prosequences for which a chaperon-like function has been suggested (Winther and Sørensen 1991). There is a speculation that the prosequence is aligned to form the conserved face interacting with the mature domain during folding. The prosequence could act as a scaffold to reduce the activation energy barrier to the native lipase.

Active production of mROL did not succeed in *E. coli* (Beer et al. 1996, 1998). Furthermore, in vitro refolding experiments showed that the prosequence was essential for the active formation of mROL (Beer et al. 1998). Also, as described above, it was shown that the prosequence of ROL was essential for the active formation and secretion of ROL by *S. cerevisiae*. Although mROL was not functionally expressed and secreted from *S. cerevisiae* by fusion with the pro-α-factor of *S. cerevisiae* (Fig. 5, lane 8), the same construct functioned in *Pichia pastoris* (Minning et al. 1998). Although the difference in expression between *S. cerevisiae* and *P. pastoris* is unknown, it could be that the pro-α-factor leader sequence helps the folding instead of the prosequence of ROL in *P. pastoris*. No significant homology is observed between the prosequence of ROL and the pro-α-factor leader sequence. Several proteins are able to be secreted from *S. cerevisiae* only by fusion with the pro-α-factor leader sequence. Since the pro-α-factor leader sequence has high solubility due to its three N-linked carbohydrate chains, it inhibits the aggregation of proteins during folding in the ER lumen (Caplan et al. 1991). However, the prosequence of ROL has only one N-linked carbohydrate chain (Takahashi et al. 1999), and the mutation of the glycosylation site in the prosequence did not affect the secretion of active ROL (date not shown). Further analysis is necessary to make clear the differences between *S. cerevisiae* and *P. pastoris*.

Based on the results presented in this study, we propose a model on the behavior of ROL in the ER lumen of *S. cerevisiae*. ROL with the wild-type prosequence is folded correctly by the function of the prosequence in the ER lumen and secreted extracellularly. On the other hand, certain deletions in the prosequence block correct folding of the mature portion, so that ROLs with the mutated prosequences are retained in the ER lumen. When the wild-type prosequence is supplied in *trans*, they can be folded to correct structures and are secreted extracellularly. However, to elucidate the structural relationship between the prosequence and mature portion, crystallographic analysis may be necessary, since to date there is no information on three-dimensional structures of the lipases involving the prosequence.

References

Beer HD, Wohlfahrt G, Schmid RD, McCarthy JEG (1996) The folding and activity of the extracellular lipase of *Rhizopus oryzae* are modulated by a prosequence. Biochem J 319:351–359

Beer HD, McCarthy JEG, Bornscheuer UT, Schmid RD (1998) Cloning, expression, characterization and role of the leader sequence of a lipase from *Rhizopus oryzae*. Biochim Biophys Acta 1399:173–180

Boel E, Huge-Jensen B, Christensen M, Thim L, Fill NP (1988) *Rhizomucor miehei* triglyceride lipase is synthesized as a precursor. Lipids 23:701–706

Caplan S, Green R, Rocco J, Kurjan J (1991) Glycosylation and structure of the yeast *MFα1* α-factor precursor is important for efficient transport through the secretory pathway. J Bacteriol 173:627–635

Chou P, Fasman GD (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol 47:45–148

Clark JL, Steiner DF (1969) Insulin biosynthesis in the rat: demonstration of two proinsulins. Proc Natl Acad Sci USA 62: 278–285

Frenken LGJ, de Groot A, Tommassen J, Verrips CT (1993) Role of the lipB gene product in the folding of the secreted lipase of *Pseudomonas glumae*. Mol Microbiol 9:591–599

Gray AM, Mason AJ (1990) Requirement for activin A and transforming growth factor-β-1 pro-region in homodimer assembly. Science 247:1328–1330

Haas M J, Allen J, Berka TR (1991) Cloning, expression and characterization of a cDNA encoding a lipase from *Rhizopus delemar.* Gene 109:107–113

Hobson AH, Buckley CM, Aamand JL, JØrgensen S, Diderichsen B, McConnell DJ (1993) Activation of a bacterial lipase by its chaperone. Proc Natl Acad Sci USA 90:5682–5686

Ihara F, Okamoto I, Akao K, Nihira T, Yamada Y (1995) Lipase modulator protein (LimL) of *Pseudomonas* sp. strain 109. J Bacteriol 177:1254–1258

Iizumi T, Fukase T (1994) Role of the gene encoding lipase activator from *Pseudomonas* sp. strain KWI-56 in *in vitro* activation of lipase. Biosci Biotech Biochem 58:1023–1027

Inouye M (1991) Intramolecular chaperone: the role of the propeptide in protein folding. Enzymes 45:314–321

Ito H, Fukuda K, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153:163–168

Ito W, Ishiguro H, Kurosawa Y (1991) A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. Gene 102:67–70

Jørgensen S, Skov KW, Diderichesen B (1991) Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes. J Bacteriol 173:559–567

Kanai T, Atomi H, Umemura K, Ueno H, Teranishi Y, Ueda M, Tanaka A (1996) A novel heterologous gene expression system in *Saccharomyces cerevisiae* using the isocitrate lyase gene promoter from *Candida tropicalis*. Appl Microbiol Biotechnol 44:759–765

Kobayashi T, Inouye M (1992) Functional analysis of the intramolecular chaperone. Mutational hot spots in the subtilisin propeptide and a second-site suppressor mutation within the subtilisin molecule. J Mol Biol 226:931–933

Kohno M, Kugimiya W, Hashimoto Y, Morita Y (1994) Purification, characterization, and crystallization of two types of lipase from *Rhizopus niveus*. Biosci Biotech Biochem 58:1007–1012

Kurooka S, Okamoto S, Hashimoto M (1977) A novel and simple colorimetric assay for human serum lipase. J Biochem (Tokyo) 81:361–369

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lee YC, Ohta T, Matsuzawa H (1992) A non-covalent NH2-terminal pro-region aids the production of active aqualysin I (a thermophilic protease) without the COOH-terminal pro-sequence in *Escherichia coli*. FEMS Microbiol Lett 71:73–77

Lippincott-Schwartz J, Bonifacino JS, Yuan LC, Klauser RD (1988) Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. Cell 54:209–229

Minning S, Schmidt-Dannert C, Schmid RD (1998) Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. J Biotechnol 66:147–156

Nagao T, Shimada Y, Sugihara A, Tominaga Y (1994) Cloning and nucleotide sequence of cDNA encoding a lipase from *Fusarium heterosporum.* J Biochem (Tokyo) 116:536–540

Neurath H (1989) Proteolytic processing and physiological regulation. Trends Biochem Sci 14:268–271

Randall LL, Hardy SJS (1989) Unity in function in the absence of consensus in sequence: role of leader peptides in export. Science 243:1156–1159

Sawai-Hatanaka H, Ashikari T, Tanaka Y, Asada Y, Nakayama T, Minakata H, Kunishima N, Fukuyama K, Yamada H, Shibano Y, Amachi T (1995) Cloning, sequencing, and heterologous expression of a gene coding for *Arthromyces ramosus* peroxidase. Biosci Biotech Biochem 59:1221–1228

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379

Shinde UP, Inouye M (1993) Intramolecular chaperones and protein folding. Trends Biochem Sci 18:442–446

Silen JL, Agard DA (1989) The α-lytic protease pro-region does not require a physical linkage to activate the protease domain *in vivo*. Nature 341:462–464

Suter U, Angst C, Tien CL, Drinkwater CC, Lindsay RM, Shooter EM (1992) NGF/BDNF chimeric proteins: analysis of neurotrophin specificity by homolog-scanning mutagenesis. J Neurosci 12:306–318

Tajima M, Nogi Y, Fukasawa T (1985) Primary structure of the *Saccharomyces cerevisiae GAL7* gene. Yeast 1:67–77

Takahashi S, Ueda M, Atomi H, Beer HD, Bornscheuer UT, Schmid RD, Tanaka A (1998) Extracellular production of active *Rhizopus oryzae* lipase by *Saccharomyces cerevisiae*. J Ferment Bioeng 86:164–168

Takahashi S, Ueda M, Tanaka A (1999) Independent production of two molecular forms of a recombinant *Rhizopus oryzae* lipase by *KEX2*-engineered strains of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 52:534–540

Tanner W, Lehle L (1987) Protein glycosylation in yeast. Biochim Biophys Acta 906:1–99

Ueda M, Mozaffar S, Atomi H, Osumi M, Tanaka A (1989) Induction and subcellular localization of enzymes participating in propionate metabolism in *Candida tropicalis*. J Ferment Bioeng 68:411–416

Voorberg J, Fontijn R, Calafat J, Janssen H, van Mourik JA, Pannekoek H (1993) Biogenesis of von Willebrand factor-containing organelles in heterologous transfected CV-1 cells. EMBO J 12:749–758

Weissman JS, Kim PS (1992) The pro region of BPTI facilitates folding. Cell 71; 841–851

Winther JR, Sørensen P (1991) Propeptide of carboxypeptidase Y provides a chaperone-like function as well as inhibition of the enzymatic activity. Proc Natl Acad Sci USA 88:9330–9334

Winther JR, Sørensen P, Kielland-Brandt MC (1994) Refolding of a carboxypeptidase Y folding intermediate in vitro by low-affinity binding of the proregion. J Biol Chem 269:22007–22013

Zhu X, Ohta Y, Jordan F, Inouye M (1987) Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. Nature 339:483–484