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Review

Expression of *Rhizopus oryzae* lipase gene in *Saccharomyces cerevisiae*

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Abstract

The extracellular production of active *Rhizopus oryzae* lipase (ROL) was carried out by the expression of the *ProROL* gene encoding a pro-form of ROL (ProROL) using prepro-α-factor in *Saccharomyces cerevisiae*. Two forms of recombinant ROL (rROL), rProROL by the expression of the *ProROL* gene and r28ROL which was a processed form of rProROL in the prosequence, were produced. Such a processing of rROL was catalyzed by the Kex2 membrane-bound endoprotease (Kex2p) in the late Golgi compartment. The ProROL and r28ROL could be produced independently as a single protein by the *Kex2*-engineered *S. cerevisiae*. Comparison of the properties of purified rROL showed that the prosequence modified some properties of ROL, and implied that the prosequence might play an physiologically important role in vivo. When only mature ROL (mROL) without the prosequence fused to the pre-α-factor leader sequence was expressed in *S. cerevisiae*, the enzyme activity was not observed in both the medium and cells. However, when the mROL was co-expressed in *trans* with the prosequence fused to the pre-α-factor leader sequence, the activity was recovered. The results showed that the prosequence may facilitate the folding of mROL, and the covalent linkage of the prosequence to the mROL was not necessary for the function. As the result of the deletion analysis at the N-terminus in the prosequence, the prosequence might work as an intramolecular chaperone. By the cell surface engineering using the gene encoding the C-terminal half of yeast α-agglutinin and the insertion of linker peptides, a novel strain displaying lipase on the cell surface was also constructed. Although *S. cerevisiae* itself is unable to utilize triolein, the transformant strain could grow on triolein as the sole carbon source. The cell surface-engineered yeast displaying ROL might be used as a potent bioctalyst.

Keywords: *Rhizopus oryzae* lipase (ROL); *Saccharomyces cerevisiae*; Prosequence; Intramolecular chaperone; Cell surface engineering

1. Introduction

In general, the bioprocesses using enzymes or microorganisms are the energy-saving processes which do not require high temperature and high pressure in comparison with chemical processes. Additionally, since enzymes have high substrate specificity and reaction specificity, the separation of aimed products is relatively easy.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) can catalyze not only hydrolysis of ester bonds under aqueous conditions but also synthesis of ester bonds, namely ester synthesis, under microaqueous conditions. These two basic processes can then be combined in a sequential fashion to give rise to a set of reactions generally termed interesterification. Depending on the

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particular starting point in terms of substrates, one may say that it is acidolysis, alcoholysis, glycerolysis, or transesterification. These reactions in chemical processes are high-energy-consuming reactions that need the high temperature and high pressure. Additionally, the resulting products are often unusable as obtained and require re-distillation to remove impurities and degradation products. Moreover, highly unsaturated heat-sensitive oils cannot be used in this process without prior hydrogenation. Since lipases have high regio- and stereo-selectivity, they can catalyze the various specific reactions, stereoselective hydrolysis of esters, stereoselective esterification of alcohols, and stereoselective and regioselective ester synthesis, for the production of optically active compounds. Due to such characteristics, systems producing a large amount of active lipases are demanded for industrial applications and understanding their fundamental characteristics for improving the properties.

The physiological properties and biochemical features of lipases have been investigated extensively. The three-dimensional structures have been presented for lipases from *Rhizomucor miehei* [1], *Rhizopus niveus* [2], *Humicola lanuginosa* [3], *Geotrichum candidum* [4], *Candida rugosa* [5], *C. antarctica* [6], *Pseudomonas glumae* [7], *Penicillium camembertii* [8], human pancreas [9], and horse pancreas [10]. Although these lipases have different amino acid sequences, they do share some common structural properties. Lipases have a catalytic center that contains a triad of three amino acids (Ser–His–Asp/Glu), like serine proteases such as chymotrypsin and subtilisin [11,12]. Lipases have a specific sequence motif, a so-called lipase–esterase consensus sequence (Gly–X–Ser–X–Gly). The serine is one of the amino acids in the catalytic center, and this sequence is identical to that in serine proteases [13]. Lipases show an α/β hydrolase-fold, which is a common three-dimensional-fold in several other hydrolases [14]. Moreover, almost all lipases have a so-called flexible lid or flap which shelters the catalytic center in solution in the resting. The lid undergoes a conformational change at the lipid–water interface, resulting in activation of lipase. This phenomenon is called "interfacial activation" [15].

Prokaryotic lipases, *Pseudomonas* lipases, need specific chaperone-like proteins assisting their correct folding in the periplasm, which were termed "modular proteins, foldases or activator proteins". These private chaperones are encoded by complete different genes that located immediately downstream of the lipase structural genes, and they could act in *trans*. The proteins are not part of the active lipase enzymes, which are composed of single polypeptide chains [16].

Eukaryotic lipases, *Rhizopus* lipases, are synthesized as pre-pro-proteins. In vivo expression and in vitro refolding experiments of *R. oryzae* lipase showed that the prosequence is necessary for the production of the active lipase [17]. Therefore, it causes the possibility that the prosequence of the lipase might act as an intramolecular chaperone.

Although many proteases require their prosequences to facilitate their folding, these findings do not constitute a rule. In addition to proteases, proteins such as growth factors [18], neuropeptides [19], hormones [20], and plasma proteins [21] also require their prosequences for their correct folding. The folding mechanisms of subtilisin E from *Bacillus subtilis* [22], α-lytic proteases from *Lysobactor enzymogenes* [23], and carboxypeptidase Y from *S. cerevisiae* [24] are well studied and gave vital information regarding the mechanism of prosequence-mediated folding. In vitro studies of them have shown that α-lytic protease and subtilisin E are folded into partially structured molten globule intermediates in the absence of their prosequences. These stable but inactive intermediates can be converted into the active conformations upon addition of their prosequences and the results suggest that the prosequences promote the folding of their proteases by direct stabilization of the rate-limiting transition state of folding. These prosequences seem to be essential only during late stages of the folding process because they help in overcoming a kinetic block in the folding pathway. However, the functional residues in the prosequences to mediate the folding are not strictly clear.

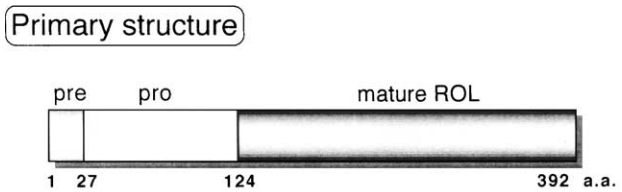
In the meantime, the expression of proteins on the cell surface of microorganisms as one of the expression systems of foreign proteins can be used for many purposes, such as the production of live vaccines, whole cell adsorbents, display and selection of antibody libraries, and whole cell biocatalysts [25]. Surface display of heterologous proteins has been described for a large number of microorganisms. In gram-negative bacteria, outer membrane proteins [26], lipoproteins [27], fimbriae [28], and flagellar proteins

[29] have all been used to immobilize heterologous proteins on the cell surface. In gram-positive bacteria, cell surface localization of several heterologous proteins is achieved by use of protein A [30], M6 protein [31], and the fibronectine-binding protein [32] as anchors. On the other hand, in eukaryotes, display of heterologous proteins is possible by using cell-wall proteins of *S. cerevisiae* [33].

The cell surface display of heterologous proteins on *S. cerevisiae* has several advantages for many reasons in comparison with that on bacteria; the surface-expressed proteins on the yeast become covalently linked to the cell-wall, the yeast is relatively resistance to autolysis because the cell-wall is rigid, and it produces only a small amount of proteases [34]. Enzymes displayed on the cell surface of yeast cells provide a way to develop a new generation of enzyme reactors [35,36]. Such an enzymatic catalyst can be simply removed from the reaction medium by filtration or centrifugation and, if necessary, can be regenerated easily. Construction of a new biocatalyst displaying lipase with activity on the cell surface of *S. cerevisiae* has developed the cell surface expression system and showed the possibility for use in application.

2. Extracellular production of active *R. oryzae* lipase (ROL) by *S. cerevisiae*

Lipase produced by *R. oryzae* (ROL) [37] comprises a signal sequence of 26 amino acids, a prosequence of 97 amino acids, and a mature lipase region of 269 amino acids, as deduced from the nucleotide sequence (Fig. 1). Among a series of triglycerides, ROL exhibited a high activity on medium chain fatty acids. Recently, structured lipids, which are triglycerides with medium chain fatty acids at their 1- and 3-positions and functional fatty acids at the 2-position, have become attractive in relation to the production of medicines and health foods. For the production of structured lipids, lipases specific to the 1- and 3-positions of triglycerides and to the medium chain fatty acids are desirable. The lipase hydrolyzes the ester bonds at the *sn*-1 and *sn*-3 positions, but not at the *sn*-2 position of triglycerides, and is, therefore, referred to a 1,3-specific lipase [37]. The 1,3-specific property has been proved particularly useful for producing structured lipids [38]. ROL seems to have some relationship with the lipases produced by *H. lanuginosa*, *R. miehei*, and *Fusarium heterosporum* [39–41]. They constitute a family of closely related proteins, exhibiting many similarities in structure and biochemical properties. However, their substrate specificities, such as fatty acid-chain length preference, are different. Therefore, further investigation of their three-dimensional structures by X-ray diffraction is necessary to elucidate the molecular basis of the structure-function relationship. For this purpose, a high-level-expression system must be developed because the amount of the enzyme produced in the original organism is small and the enzyme is heterogeneous. Although ROL has already been expressed in *Escherichia coli*, enzymatically inactive and insoluble aggregates were obtained [42]. On the other hand, the functional expression and secretion of active lipases from *G. candidum* and *F. heterosporum* have been reported using *S. cerevisiae* as the host



Presequence (26 a.a.) is for protein localization to the endoplasmic reticulum and secretion.
Prosequence (97 a.a.)
The mature lipase (269 a.a.) is secreted as a 30 kDa protein.

Fig. 1. Structure of ROL.

[43,44]. Furthermore, a novel and strong heterologous gene expression system in *S. cerevisiae* using the 5′-upstream region of the isocitrate lyase gene (*UPR-ICL*) from the alkanotrophic yeast, *C. tropicalis* has been developed [45]. *UPR-ICL*-mediated transcription is repressed by glucose, but strongly induced by non-fermentable carbon sources such as glycerol, acetate or ethanol or under the conditions of derepression [46,47]. The *UPR-ICL* is one of the powerful promoters in *S. cerevisiae*, similar to *GAL1* or *GAL7*. Using the expression plasmids of ROL, production of the active enzymes was assessed by a halo formation around the colony on YPD agar plates containing an emulsion of tributyrin [48]. The importance of the prosequence for refolding of ROL from inclusion bodies was also suggested in *E. coli*. The prosequence may play important roles in the folding and transport of the precursor protein in the cellular secretory pathway. The requirement of the prosequence of ROL for efficient expression and secretion of ROL was confirmed. The highest extracellular lipase activity of 2880 U/l (28.0 mg/l) was obtained in the culture supernatant at 120 h of cultivation.

The rROL purified from the culture supernatant of the transformant yielded a single protein band on native-PAGE, and the activity staining on native-PAGE confirmed that the protein possessed lipase activity. On SDS-PAGE, this final preparation gave two bands corresponding to 46 and 35 kDa. The N-terminal amino acid sequence of the 46 kDa protein was Val–Pro–Val–Ser–Gly–Lys–Ser–Gly–Ser–Ser, corresponding to N-terminal amino acids of the prosequence of ProROL. On the other hand, the N-terminal amino acid sequence of the 35 kDa protein was Asp –Asp – Asn– Leu –Val – Gly –Gly– Met– Thr–Leu which corresponded to the C-terminus of the prosequence of ProROL. From these results, the 46 kDa protein was a precursor with the complete prosequence (rProROL), while the 35 kDa protein was mROL attached with 28 amino acids of the C-terminal part of the prosequence (r28ROL) (Fig. 2).

The substrate preference of rROL, composed of rProROL and r28ROL, was examined by titrimetric assay using a series of simple triglycerides. The rROL reacted well on tricaprylin (C8), tricaprin (C10), trilaurin (C12), and trimyristin (C14), but was less reactive on tributyrin (C4) and tripalmitin (C16), etc. among a series of triglycerides.

Both unprocessed and partially processed forms, as in this case, have been demonstrated for several heterologous proteins secreted by *S. cerevisiae*. This processing could be catalyzed by a protease like Kex2 endoprotease in the late Golgi compartment. Such assembly of unprocessed and partially processed proteins derived from the same expression products, as demonstrated by native-PAGE, may occur as a result of some non-covalent force, for example, the interaction between glycosyl side chains.

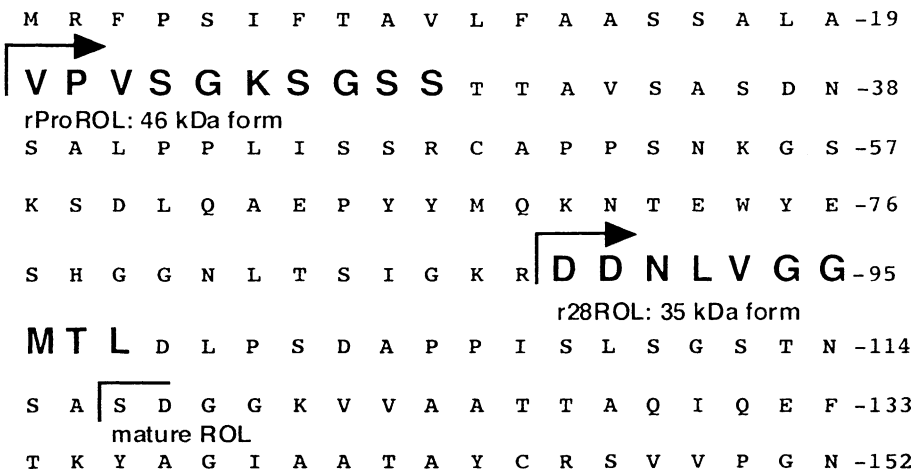


Fig. 2. N-terminal amino acid sequence of rROL produced by *S. cerevisiae*. Large and bold letters represent the N-terminal amino acid sequences determined by a sequencer. In this figure, a part of the presequence mentioned in Fig. 1 is also represented.

The high-level expression and secretion of *R. oryzae* lipase by *S. cerevisiae* in its active form, unlike the case in *E. coil*, was carried out. This expression system is useful not only for analysis of the structure–function relationship of the enzyme, but also for improvement of its functions and properties by protein engineering [48].

3. Analysis of processing of recombinant *R. oryzae* lipase precursors in *S. cerevisiae*

The post-translational proteolytic modification in the secretory pathway is essential for maturation of precursors of many secretory proteins in eukaryote. Enzymes which cleave precursors at the carboxyl side of pairs of basic residues or similar sites are characteristic of subtilisin-type serine proteases [49,50]. The Kex2 endoprotease (Kex2p) of *S. cerevisiae* is a prototype of this family and was discovered through the analysis of mutant strains lacking the post-translational processing of the precursors of the mating pheromone α-factor and the killer toxin [51,52]. Kex2p is a Ca^{2+}-dependent and membrane-bound endoprotease, which localizes in the late Golgi compartment, and initiates α-factor maturation by cleaving the spacer region at Lys–Arg dibasic site [51]. Kex2p consists of pre signal sequence, prosequence, subtilisin domain, a Ser/Thr-rich region, a transmembrane domain (TMD), and a C-terminal cytosolic tail [53]. The TMD and the C-terminal cytosolic tail are essential for localization in the late Golgi compartment [54]. From the deletion analysis of C-terminal part of Kex2p, it has been shown that the Ser/Thr-rich region, the TMD, and the C-terminal cytosolic tail are not required for enzymatic activity [55]. Since Kex2p has a high site-specificity, it has been shown in vitro that Kex2p can be one of the useful tools to cleave recombinant fusion proteins with poly His tags, which are introduced for the simple and rapid purification of expressed proteins by affinity chromatography on chelated metal ion columns [56].

Many successful examples have been reported on the secretion system of fusion proteins with yeast prepro-α-factor leader sequence, which bears the Kex2p cleavage site. When these proteins are correctly processed at the target site, the reaction of Kex2p can be rate-limiting in processing and secretion. In fact, a part of secreted proteins still having the pro-α-factor leader sequence were detected in the culture supernatant. Therefore, the improvement of the expression system, for example, the co-expression of the Kex2p-encoding gene is requested for the complete processing.

A *Kex2-613* gene expression product, which is the soluble form of Kex2p (sKex2p) lacking C-terminal 201 amino acids corresponding to the Ser/Thr-rich region, the TMD and the C-terminal cytosolic tail, was secreted in a considerable quantity into the culture medium and still retained the enzymatic activity [57]. Each gene encoding Kex2p or sKex2p was expressed under the control of *UPR-ICL*. To demonstrate whether Lys(−30)–Arg(−29) in the prosequence of ROL was a recognition site by Kex2p, a mutation was introduced into the gene coding for Arg(−29) to Lys. According to the specificity of Kex2p, the degree of the cleavage of ROL might be decreased by this mutation. Analysis of the secreted proteins in the culture supernatant using the *kex2* strain showed that r28ROL (35 kDa protein) completely disappeared, and that only rProROL (46 kDa protein) was observed [58]. These results strongly indicated that Kex2p was responsible to the cleavage. In yeast, Kex2p, Yap3p, and Mkc7p have been reported as endoproteases which cleave the proteins at dibasic amino acids [59,60]. However, the protein secreted from the *kex2* cells was only rProROL, indicating that Kex2p is a central enzyme of the processing in the secretory pathway. In fact, the latter two endoproteases can complement the function of Kex2p only in the case of their excess expression. Furthermore, in the case of the mutated *ROL* gene, the major protein secreted was rProROL, although a small amount of r28ROL was observed. These results demonstrated that Lys(−30)-Arg(−29) in the prosequence of rProROL was the recognition sequence. Kex2p secreted by deleting the C-terminal amino acids for membrane anchoring was used in vitro for cleaving α-factor leader fusion proteins and His-tag fusion proteins bearing the Kex2p cleaving site. Unlike such experiments in vitro, the complete cleavage of rProROL was carried out in situ when the *ROL* gene and the gene encoding sKex2p were co-expressed. In this case, most of the target protein might be cleaved by sKex2p in the secretory process, probably, in the closed area like the Golgi apparatus and/or secretory vesicles, although there is a

possibility that a part of the target protein is processed after secreted into the culture medium. In fact, when the active sKex2p was co-produced with the target protein, the processing of rProROL was completely performed.

4. Characterization of recombinant *ROL* produced by *KEX2*-engineered strains of *S. cerevisiae*

The production of two different molecular forms of recombinant lipases using the *kex2* strain and the strain harboring the gene encoding a soluble form of Kex2 endoprotease (sKex2p) became to be possible. To individually purify these two forms of rROLs (rProROL and r28ROL), such two *KEX*-engineered host cells were used. The rProROL was produced as a single protein by the *kex2* strain, and secreted into the culture supernatant. On the other hand, r28ROL was prepared from the culture supernatant obtained by co-expression of sKex2p. Based on the specific lipase activity of each purified rROL, the amounts of the enzymes secreted were estimated as 78.8 mg/l broth for rProROL and 38.3 mg/l broth for r28ROL [61].

The result obtained from the determination of the molecular masses of rProROL and r28ROL on a Sephadex G-200 column suggested that the native rROL was a monomeric form. The rProROL has three potential consensus sequences of *N*-glycosylation (*N*-X-S/T, where X is any amino acids except for proline) in which one is located only in rProROL. The rROLs were treated with endoglycosidase H (Endo H) to hydrolyze the *N*-linked carbohydrate chains. After this treatment, the decrease (3 kDa) in molecular mass was observed in rProROL, whereas, the molecular mass of r28ROL did not change [61].

The rProROL had its highest activity at 30 °C, while r28ROL at 25 °C. The optimum reaction temperature of r28ROL was slightly lower than that of rProROL. Thermostability was examined by incubating each rROL at various temperatures for 30 min and measuring the residual activity at 30 °C. *T50* (the temperature resulting in 50% loss of the original activity) was about 55 °C for rProROL and 40 °C for r28ROL, respectively. As rProROL was glycosylated but r28ROL was not, the effect of glycosylation on increased thermostability was examined by treatment with Endo H. However, the stability of rProROL was not affected by de-glycosylation. Lipases secreted by *S. cerevisiae* are often glycosylated [62]. For example, the lipases of *P. camembertii* and *G. candidum* produced by *S. cerevisiae* were modified by *N*-linked glycosylation [63,64]. The ROL has three potential *N*-glycosylation sites. One site is in the prosequence portion, and the others are in the mature portion. Since the molecular mass of rProROL but not r28ROL was decreased by the Endo H treatment, only one glycosylation site in the prosequence of rProROL was shown to be modified by an *N*-linked carbohydrate chain. The effect of glycosylation on thermostability has been investigated using several proteins secreted by *S. cerevisiae* [65,66]. As the thermostability of rProROL was not decreased by the Endo H treatment, the higher thermostability of rProROL could not be due to the *N*-glycosylation. The difference in thermostability was also observed when ROL was expressed and refolded in *E. coli*. Since proteins expressed in *E. coli* are not modified by glycosylation, the thermostability of rProROL might be obtained by the presence of the prosequence. These results indicated the possibility that the prosequence itself, especially the region cleaved by sKex2p, stabilized the overall conformation of ROL [61].

The rProROL was stable between pH 2.2 and 8.0, and r28ROL between pH 3.0 and 10.0. The rProROL was more stable than r28ROL under acidic conditions (pH 2.2), and r28ROL was more stable than rProROL under alkaline conditions (pH 9.0 and 10.0). The substrate specificity was determined by the spectrophotometric method using *p*-nitrophenyl esters (*p*-NE) as substrates. The rProROL had its highest esterase activity toward *p*-nitrophenyl laurate (C12) and lower activity toward *p*-nitrophenyl acetate (C2) and *p*-nitrophenyl stearate (C18), whereas, r28ROL had its highest esterase activity toward *p*-nitrophenyl caprylate (C8) and lower activity toward *p*-nitrophenyl acetate (C2). A remarkable difference in substrate specificity (in the esterase activity) between rProROL and r28ROL was observed on *p*-nitrophenyl stearate (C18). The low activity of rProROL might be due to steric hindrance between the prosequence and a substrate with a long chain. Furthermore, interestingly, the specific esterase activity of rProROL toward *p*-nitrophenyl esters was almost 10–20 times lower than that of r28ROL, although lipase activity

was not so different when measured with Lipase Kit S (Dainippon Pharmaceutical Co. Ltd., Osaka). These results indicated that the prosequence had some effect on the esterase activity [61].

5. Function of the prosequence of *ROL* as an intramolecular chaperone in *S. cerevisiae*

When ROL was expressed in *E. coli* [17,67], the prosequence of ROL was shown to have the function to protect the cells from the toxic activity of the mature portion. Additionally, the prosequence was necessary for the formation of the active ROL in vivo and in vitro, and it could act in *trans* in vivo. In *E. coli*, the prosequence of ROL might play a same manner as the prosequence of bovine pancreatic trypsin inhibitor (BPTI) [17] in folding these mature portions. These prosequences seem to facilitate the folding by providing an intramolecular thiol-disulfide reagent [68]. However, the function in eukaryotic cells is not clear yet.

When the mature portion of ROL (mROL) fused to the pre-α-factor leader sequence was expressed in *S. cerevisiae*, the lipase activity was not detected in both the culture supernatant and the cell homogenate, although the activity was detected in both fractions when the mROL having the prosequence at the N-terminus (ProROL) was expressed in *S. cerevisiae* (*cis*-expression) [69]. Therefore, the prosequence might support the correct folding of its mature portion in *S. cerevisiae*. In the function of the prosequence of the subtilisin E from *B. subtilis* on its correct folding, the covalent linkage between its mature portion and the prosequence was not necessary. To examine whether or not the covalent linkage between the prosequence of ROL and mROL is essential for the formation of the active ROL, the prosequence and mROL were co-expressed in *trans* (individually and coincidentally) in *S. cerevisiae kex2* strain. The activity was not detected when only mROL was expressed, whereas the significant activity was detected in the culture supernatant and in the cells when mROL was co-expressed in *trans* with the prosequence. These results showed that the covalent linkage between the prosequence and mROL was not essential for the formation and secretion of the active ROL [69].

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. 3) [69]. The genes encoding the deleted prosequences fused to the pre-α-factor leader sequence were co-expressed in *trans* with mROL in *S. cerevisiae kex2* strain. The sufficient activity was still observed in the culture supernatants of the mutants with

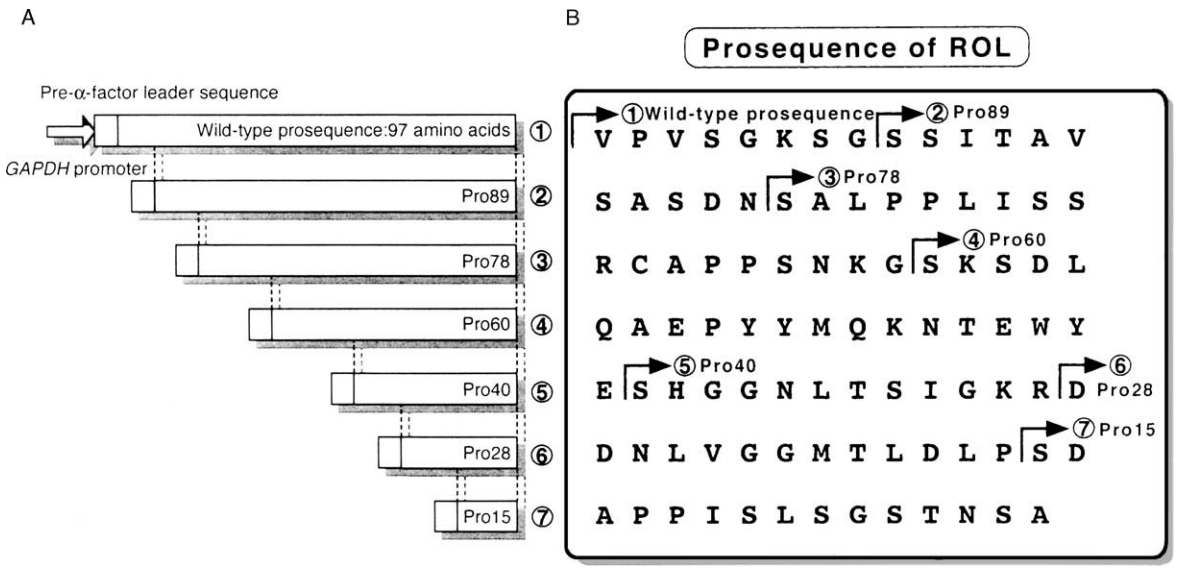


Fig. 3. Schematic representation (A) and amino acid sequences (B) of the N-terminal deletion of the prosequence of ROL.

the prosequences deleted 8 (Pro89) and 19 residues (Pro78) from the N-terminus. The activity was decreased by the deletion of 8 residues, and recovered by the deletion of 19 residues, whereas the activity in the culture supernatant was markedly reduced by deletion of more than 37 residues (Pro60). However, the sufficient activity, in the case of Pro60, was observed in the cells. Deletion of more than 57 residues (Pro40) markedly reduced the activity both in the cells and in the culture supernatant [69]. These results indicated that the region from 38 (Pro60) to 57 (Pro40) residues was necessary for the formation of the active ROL.

From the results of Western blot analysis [69], it can be confirmed that the region from 20 to 37 residues of the prosequence is essential for the secretion of the mature portion, and that the region from 38 to 57 residues is necessary for the correct folding to form the active ROL. As a conclusion, the region from 20 to 57 residues would be significant for in vivo folding and secretion of active mROL, and the region from 38 to 57 residues in that portion might play a role as an intramolecular chaperone (Fig. 4). The possible function of the prosequence of ROL is clear as an intramolecular chaperone on the secretion and the formation of its mature portion in the expression system using *S. cerevisiae* as a host [69].

In general, secretory proteins that are not folded correctly are retained in the ER lumen. 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, the correct folding of mROL in *trans* could be performed.

The prosequence playing the role as an intramolecular chaperone has also been observed in several proteases, such as subtilisin [22], α-lytic protease [23], aqualysin [70], and carboxypeptidase Y [24]. 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 [71]. The prosequence of ROL has no significant similarity to the prosequences 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 the mature portion; 19.7% (15Asp, 9Glu, 15Lys, 7His and 9Arg; net charge, +9). It is quite interesting that the prosequences of lipases and proteases have the similar function on folding, because they are functionally, structurally and also evolutionarily different enzymes except for that the lipases have catalytic amino acids (Ser–His–Asp), like serine proteases, such as chymotrypsin and subtilisin.

Despite the putatively functional role of the prosequences of proteases in folding, very little is known about the specific regions important for this function. Kobayshi and Inouye [72] identified three hydrophobic regions within the subtilisin E prosequence from *B. subtilis*, which plays an important role in the

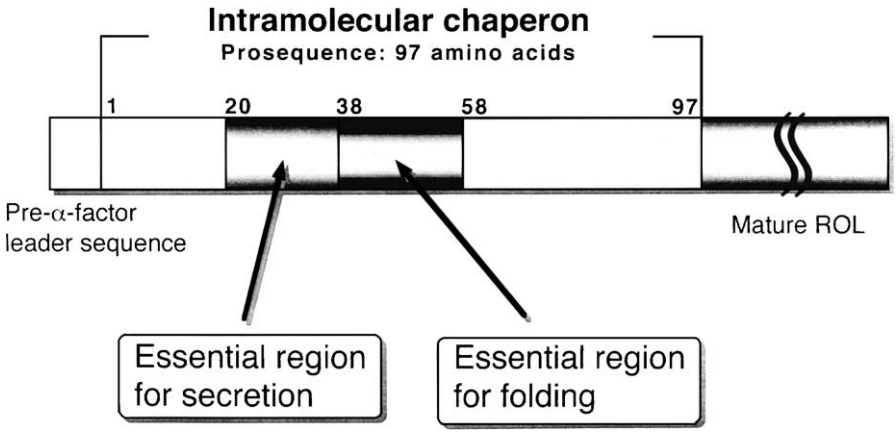


Fig. 4. Role of the prosequence of ROL as the intramolecular chaperon.

folding of prosubtilisin, presumably by interaction of these regions with a portion of the mature enzyme.

In the case of ROL, Beer et al. [17] presumed that cysteine-30 (Cys-30) in the prosequence might play a key role in facilitating the folding of the enzyme and work as an intramolecular-disulfide reagent, analogously to the role of the cysteine residue in the prosequence of pro-BPTI [68]. Although the secretion of the active mROL was greatly reduced by the deletion of the residues from 20 to 37 containing Cys-30 in the prosequence, the activity was still observed in the cell. In addition, the mutation from Cys-30 to serine did not affect the activity in both extra- and intra-cellular fractions. On the other hand, the activity in the cell was greatly reduced by the deletion of residues from 38 to 57 in the prosequence, suggesting that the residues from 38 to 57 are essential for the formation of the active ROL in vivo, although only the fragment from 20 to 57 residues was functional in the co-expression of mROL with the fragment of the prosequence. The secondary-structure analysis using the method of Chou and Fasman [73] predicted that the region from 38 to 57 residues constitutes one α-helical structure. This is a common structural feature of prosequences for which chaperone-like function have been suggested [71]. 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.

The active production of mROL was not succeeded in *E. coli* [17,37]. Furthermore, in vitro refolding experiment showed that the prosequence was essential for the active formation of mROL. Also, as described above, it was shown that the prosequence of ROL was essential for the formation and the secretion of active ROL by *S. cerevisiae* [69].

6. Application of lipase by cell surface engineering

Recently, "cell surface engineering", which enables host cells to obtain new functions such as the abilities to utilize starch and cellulosic materials, has been progressed [35,36]. In yeast systems, a secretion signal sequence and the glycosylphosphatidylinositol (GPI)-anchor attachment signal sequence of the native cell-wall anchored protein, α-agglutinin, are genetically fused to N-terminal and C-terminal regions of the target protein, respectively. The α-agglutinin is a mannoprotein involved in the sexual adhesion of mating type α *S. cerevisiae* cells with mating type a cells. On the secretory pathway, the GPI-anchor is attached to the fused protein after digesting the GPI-anchor attachment signal sequence, and the protein is covalently linked to cell-wall through the GPI-anchor. The system that immobilizes an enzyme genetically on the cell surface has a lot of advantages, easy to reproduce displayed biocatalysts, easy to separate products from catalyst, and so on. As various kinds of proteins and peptides can be displayed on the yeast cell surface, this system is expected to lead preparing tailor-made biocatalysts. From these advantages, "cell surface engineering" could be applied in a lot of fields in biotechnology.

Display of lipase on the yeast cell surface is of great interest in constructing a novel biocatalyst. Lipase-displaying yeast might be applied even to the established processes. Further, since *S. cerevisiae* and lipase of *R. oryzae* are both confirmed in human oral use, this biocatalyst could be suitable for food-producing processes. Although cell surface display of lipase from *H.* (*Thermomyces*) *lanuginosa* and cutinase from *F. solani* f. sp. *pisi*, fused to α-agglutinin, was tried before and the enzymes were detected immunologically on the cell surface of *S. cerevisiae*, the displayed enzymes exhibited no activity towards an emulsion of olive oil, even though a very low enzymatic activity was demonstrated towards *p*-nitrophenyl butyrate [25]. The lack of the enzymatic activity was assumed to be caused by hindrance of lipid-binding to the enzymes, because analysis of the three-dimensional-structure of *Rhizopus* lipase indicated that the active site is located at the C-terminal region.

The spacer-mediated display of ROL (ProROL) on the yeast cell surface was examined (Fig. 5) [74]. The ROL-displaying cells surely exhibited a high lipase activity towards triolein. The insertion of the spacer (linker peptides composed of a Ser/Gly repeat sequence) with different lengths between ROL and the C-terminal half of α-agglutinin was demonstrated to contribute the increase in the lipase activity displayed on the cell surface.

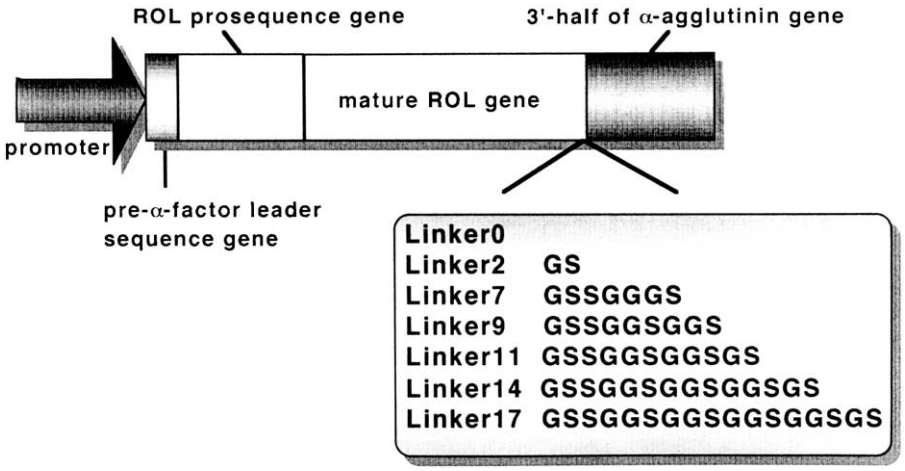


Fig. 5. Gene structure for display of ROL on the yeast cell surface.

To confirm the localization of ROL on the yeast cell surface, immunofluorescent labeling of the cells was conducted with the anti-ROL antiserum as the primary antibody and the fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG as the second antibody. These results indicated that ROL expressed was localized on the surface of the respective cells. The pellet of the ROL-displaying cells exhibited the lipase activity to convert 2,3-dimercaptopropan-1-ol tributyl ester (BALB) to 2,3-dimercaptopropan-1-ol, while the activity in the culture supernatant was not detectable. Insertion of the linker peptides between ROL and α-agglutinin resulted in the increase of the enzyme activity: Therefore, it can be concluded that ROL displayed on the cell-wall exhibited the enzyme activity towards BALB, and the linker peptides enhanced the enzyme activity [74].

ROL-displaying cells could hydrolyze the emulsion of triolein (C18:1). The transformants were cultivated aerobically in the medium containing 1% (w/v) triolein as a sole carbon source. The ROL-displaying cells could grow on triolein as a sole carbon source, while the control cells could not. To confirm the hydrolysis of triolein, the oil fractions in the culture media were analyzed by thin-layer chromatography and gas chromatography. The 1,3-diolein, 1,2-diolein, and oleic acid were detected in the oil fractions of the culture media of the ROL-displaying cells, although no hydrolysate was observed on the control cells, suggesting that the enzyme displayed on the cell surface reacted with the emulsion of triolein and hydrolyzed ester bonds. Each cell displaying ROL on the cell surface hydrolyzed triolein, and there was a tendency to become the activity higher as linker peptides were longer. When triolein is hydrolyzed by the lipase displayed on the yeast cell surface, the primary product is oleic acid. In fact, cell growth in 0.96% (w/v) oleic acid-containing medium reached the same level as that in 1% (w/v) triolein-containing medium [74].

Insertion of spacers between ROL and the C-terminal half of α-agglutinin gave us an attractive information. The linker peptide sequences consisting of Gly/Ser repeats are often used because of their conformational flexibility and hydrophilic feature [70,75–77]. Linker peptides gave a significant effect to enhance the hydrolyzing activity towards BALB and triolein of the ROL-displaying cells. The spacer on the cell surface of the ROL-displaying cells seems to contribute to the separation of the active moiety from the cell-wall binding moiety. As the fatty acid-binding site of ROL is located near the C-terminal, the insertion of the spacer would help the ROL-α-agglutinin fusion protein to make a space for the access of substrates. This tendency was clear for BALB. It should be noted that longer linker peptides (17 amino acids) yielded a high activity towards triolein, which was higher than that of the cells secreting ROL [74]. This

difference in the enzyme activity may be resulted from the necessity of the enzyme flexibility toward bulky and soluble BALB and insoluble triolein, that is, there would exist the best length of the linker peptides depending on the kinds of substrates.

The cell surface display eliminates a purification step of the enzyme, leading to the cost-down in the preparation of the biocatalyst. In addition, cell surface-displayed ROL exhibited the same and/or higher level of the enzyme activity compared to extracellularly produced ROL towards triolein. Thus, the processes with biocatalysts prepared by "cell surface engineering" have a possibility to substitute for the established processes. As "cell surface engineering" is a newly developed method to construct biocatalysts conveniently, there is a lot of possibility of expansion. The results demonstrated here would give further applications in biotechnology [78].

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