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Effect of the truncation of the C-terminal region of Kex2 endoprotease on processing of the recombinant *Rhizopus oryzae* lipase precursor in the co-expression system in yeast

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Abstract

The *Saccharomyces cerevisiae* Kex2p is a membrane-bound endoprotease and cleaves at a C-terminal site of Lys–Arg of the α-factor precursor in late Golgi compartment. The complete cleavage of a secreted recombinant *Rhizopus oryzae* lipase (ROL) precursor (rProROL) having an internal Lys–Arg has been examined by the co-expression of the *KEX2* gene. The cleavage of rProROL was not sufficient. The co-expression system of the truncated gene encoding the soluble form of Kex2p (sKex2p) lacking C-terminal 201 amino acids containing the transmembrane domain (TMD) was constructed. sKex2p co-expressed was detected in the culture medium and the carboxyl site of Lys(-30)–Arg(-29) in the prosequence of rProROL was completely cleaved. These results revealed that on the process of secretion, including in the Golgi apparatus and secretion vesicles, attack of sKex2p to the produced protein occurred more efficiently than that of Kex2p. The cells having the co-expression system of the gene encoding sKex2p will be useful for production of foreign proteins designed to cleave the internal Lys–Arg site for their activation or for the application of the prepro-α-factor leader sequence.

Keywords: Kex2 endoprotease; *Rhizopus oryzae* lipase: Processing; Transmembrane domain; Co-expression; Yeast

1. Introduction

The secretion of proteins into culture media is a convenient method to obtain large amounts of recombinant foreign proteins in a relatively pure form without contamination of cellular proteins. The yeast, *Saccharomyces cerevisiae*, is one of the desirable hosts for this purpose because it can grow to a high density in cheap media. Moreover, since it is a eukaryotic microorganism, secreted proteins are exposed to a number of post-translational modifications which share common mechanisms to higher eukaryotes, including disulfide bridge formation, glycosylation, and proteolytic processing [1,2].

The post-translational proteolytic modification in the secretory pathway is essential for maturation of precursors of many secretory proteins in eukaryote [3]. Enzymes which cleave precursors at the carboxyl side of pairs of basic

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residues or similar sites are characteristic of subtilisin-type serine proteases [4,5]. 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 M_1 killer toxin [6,7]. 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 [6,8–11]. Kex2p consists of pre-signal sequence, prosequence, subtilisin domain, a Ser/Thr-rich region, a transmembrane domain (TMD), and a C-terminal cytosolic tail (Fig. 1A) [12]. The TMD and the C-terminal cytosolic tail are essential for localization in the late Golgi compartment [13]. 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 [8]. A *KEX2Δ613* 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 [12,14].

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 [15].

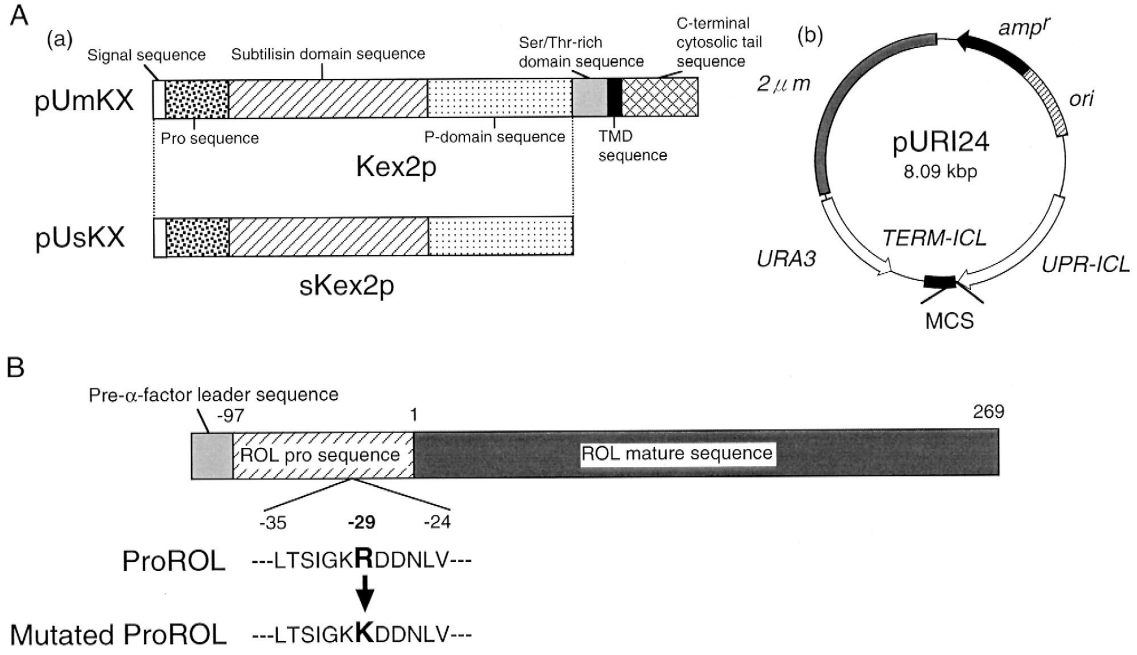

Fig. 1. Structure of the Kex2p-encoding gene and physical maps of the expression plasmids for the production of Kex2p and sKex2p (A), and the protein structure of rProROL and mutated rProROL (B). (A) The *KEX2* gene and the *KEX2Δ613* gene (a) were inserted into the multicloning site (MCS) of plasmid pURI24 (b) between *UPR-ICL* and *TERM-ICL* of isocitrate lyase gene derived from *C. tropicalis* pK233. P-domain is represented in Ref. [12] and TMD means the transmembrane domain. The plasmid (b) contains the following components: *ori*, *amp*^{r}, *2μm*, *URA3*, and MCS. The plasmid containing complete ORF of the *KEX2* gene is pUmKX and the plasmid containing the *KEX2Δ613* gene deleted the region including the membrane anchoring is pUsKX. (B) The *ROL* gene and the mutated *ROL* gene at a processing site in the pro sequence fused with the gene of pre-α-factor leader sequence, and inserted into pWI3 as described in Section 2. The N-terminal amino acid of the mature sequence was defined as the position, +1.

Many successful examples have been reported on the secretion system of fusion proteins with yeast prepro-α-factor leader sequence, which bear 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 [11,16]. In fact, a part of secreted proteins still having the pro-α-factor leader sequence was detected in the culture supernatant [16]. Therefore, the improvement of the expression system, e.g., the co-expression of the Kex2p-encoding gene, is requested for the complete processing. In the previous paper [17], we reported that on the production of *Rhizopus oryzae* lipase (ROL) using the presequence of α-factor of *S. cerevisiae*, the precursor protein (rProROL) and the protein cleaved at Lys(-30)–Arg(-29) in the prosequence (r28ROL) were detected in the culture supernatant. Here we describe the effect of the co-expression of Kex2p or sKex2p on the processing of the recombinant ROL precursor.

2. Experimental

2.1. Strains and media

Escherichia coli DH5α[F^-, *endA1*, *hsdR17*(r_k^-,m_k^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *ΔlacU196*, *ϕ80dlacZΔM1*] was used as a host for recombinant DNA manipulation. *S. cerevisiae* strain MT8-1 (MAT*a*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) [18] was used as the host for the protein production. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) containing 0.1% glucose and 50 μg/ml ampicillin. Yeast was precultivated in SD medium [2% glucose and 0.67% yeast nitrogen base without amino acid (Difco, Detroit, MI, USA) with appropriate supplements] and cultivated aerobically in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or YPDBC medium which is YPD medium containing 50 mM Bis-tris and 1mM $CaCl_2$, adjusted to pH 7.2 with 1 N HCl.

2.2. Construction of the plasmid

The plasmid for the expression of the Kex2 endoprotease (Kex2p)-encoding gene was constructed as follows: the 2.45-kbp open reading frame of the *KEX2* gene was amplified by the polymerase chain reaction (PCR) with the genomic DNA of *S. cerevisiae* MT8-1 as a template. The genomic DNA was isolated by the method of Hoffman and Winston [19]. The primers used were KexOp1 (5′-CCCATGTCGACATGAAAGTGAGGAAATATATTA--3′) and KexOp2 (5′-ACATCTCGAGTCACGATCGTCCGGAAGATGGAGG-3′). The amplified fragment was digested by *Sal*I and *Xho*I (underlined), and then introduced into the multicloning site between *UPR-ICL* and *TERM-ICL* of pURI24, which has a *Pvu*II/*Bam*HI fragment containing *UPR-ICL* and *TERM-ICL* of pWI3 [20], instead of the *Pvu*II/*Bam*HI fragment containing the *GAL7* promoter of pMT34(+3) [18]. The resulting expression plasmid for the *KEX2* gene was named pUmKX. The plasmid for the expression of the secretion form of Kex2p (sKex2p) was constructed as follows: the 1.84-kbp *KEX2Δ613* gene encoding sKex2p was obtained by PCR by deleting 603-bp in the 3′-terminal region of the *KEX2* open reading frame gene which encodes the C-terminal 201 amino acids of Kex2p [21]. The deleted fragment was amplified by PCR with pUmKX as a template. The primers used were KexOp1 and sKexp (5′-TAGCCTCGAGTTATTCAACCTCCTCTTTATCGTTTCCAAAGACG-3′). The amplified fragment was digested by *Sal*I and *Xho*I (underlined), and then introduced to pURI24. The resulting expression plasmid for sKex2p was named pUsKX. rProROL mutated Arg(-29) to Lys in the prosequence was obtained by the site-directed mutagenesis [22] using the primer PrRLK (5′-CTGACATCCA TCGAAAGAAAGATGACAATTTGGTTG-3′) for the mutation (the mutated sequence underlined). The mutated *ROL* gene was cut by *Sal*I and *Eco*RI, and the resulting fragment was introduced into pUC19. After the plasmid was

cut by *Sal*I and *Xho*I, the *Sal*I/*Xho*I fragment was introduced into pWI3 [20]. The resulting plasmid for expression of the mutated ROL was named pWRL2K. The plasmid pWRL2 [17] was used for the expression of *R. oryzae* lipase in *S. cerevisiae*.

2.3. Disruption of the KEX2 gene

A fragment containing the *KEX2* gene and the noncoding region was amplified by PCR with genomic DNA as a template. The primers used were KexDp1 (5′-CACAGGATCCAATCGCCTCCGTTAGAATTTGCCTCC-3′) and KexDp2 (5′-TATCGTCGACACATATACGTATACGTATGAGAGAG-3′). The PCR product was digested with *Bam*HI and *Sal*I (underlined), and cloned into pUC19. The resultant plasmid was digested with *Eco*RV on *KEX2*, and then ligated with the *HIS3* gene from pRS403 as a selectable marker [23]. The plasmid obtained was linearized by digestion with *Bam*HI and *Sal*I to transform the parent strain, MT8-1. Transformants with the *HIS3*$^+$ phenotype were isolated, and disruption of the chromosomal *KEX2* gene was confirmed by Southern blot analysis. The *kex2* strain was designated as strain KDH3.

2.4. Preparation of secreted proteins

The culture supernatant of transformants was obtained by centrifugation at $4000 \times g$ for 10 min at 4°C, and then two-fold volume of acetone (−20°C) was added to the supernatant. After the mixture was left overnight at −20°C, insoluble materials were collected by centrifugation at $12{,}000 \times g$ for 30 min at −10°C. The pellet was washed with 70% acetone (−20°C) and dried *in vacuo*. The dried pellet was dissolved in water, and the solution was concentrated and desalted using a Centriplus-30 (Amicon, Beverly, MA, USA).

2.5. Measurement of protein

Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA) using bovine serum albumin as a standard.

2.6. Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [24]. After the electrophoresis on a 10% (w/v) or 12.5% (w/v) SDS gel, proteins were stained with Coomassie Brilliant Blue R-250 and molecular masses were estimated by using the following standard proteins (Amersham Pharmacia Biotech, Uppsala, Sweden): phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa.

2.7. Analysis of N-terminal amino acid sequence

The N-terminal amino acid sequences were determined by automated Edman degradation using a Protein Sequencer 610A (Applied Biosystems, Foster City, CA, USA). Each sample was separated by SDS-PAGE and electroblotted onto a sheet of ProBlott (Applied Biosystems). After staining with Coomassie Brilliant Blue R-250, the bands of proteins were cut to use for the sequencing.

3. Results

3.1. Construction of the expression plasmids

For the expression of the *KEX2* gene, the open reading frame was amplified by PCR with the genomic DNA of *S. cerevisiae* as a template. To secrete the endoprotease (sKex2p) from *S. cerevisiae*, the 603-bp fragment of 3′-terminal region of the *KEX2* gene (*KEX2Δ613*), which is the DNA fragment encoding 201 amino acids

corresponding to the Ser/Thr domain, the TMD, and the C-terminal tail [21], was deleted. Each gene encoding Kex2p or sKex2p was expressed under the control of *UPR-ICL*, the upstream sequence of isocitrate lyase gene of *Candida tropicalis* pK233 functional in *S. cerevisiae* [20], on the multicopy plasmid pURI24 (Section 2) which has a *URA3* selectable marker (Fig. 1A). The expression plasmids for Kex2p and sKex2p were named pUmKX and pUsKX, respectively. 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 encoding for Arg(-29) to Lys (Fig. 1B). According to the specificity of Kex2p shown by Mizuno et al. [25], the degree of the cleavage of ROL might be decreased by this mutation. Each *ROL* gene and the mutated *ROL* gene were fused with the pre-α-factor leader sequence instead of original signal sequence of ROL to attain efficient secretion from *S. cerevisiae*, and expressed under the control of *UPR-ICL* on the plasmid pWI3 [20] which has a *TRP1* selectable marker (Fig. 1B). The expression plasmid for the mutated *ROL* gene was named pWRL2K.

3.2. Processing of rProROL

The parent strain, MT8-1, carrying the plasmid pWRL2 secreted two molecular forms of ROL having different molecular masses (46 and 35 kDa) (Fig. 2, lane 2). The N-terminal amino acid analysis of these recombinant ROLs revealed that 46 kDa protein was a precursor ROL (rProROL) and 35 kDa protein was a cleaved form in the prosequence (r28ROL) [17]. The cleavage seemed to be mediated by Kex2p because the N-terminal amino acid of r28ROL was Asp just after Lys–Arg [17], a recognition site of Kex2p [6]. When the plasmid pWRL2 harboring the *ROL* gene was introduced into the *kex2* strain, KDH3, and the secreted proteins in the culture supernatant were analyzed by SDS-PAGE, r28ROL (35 kDa protein) completely

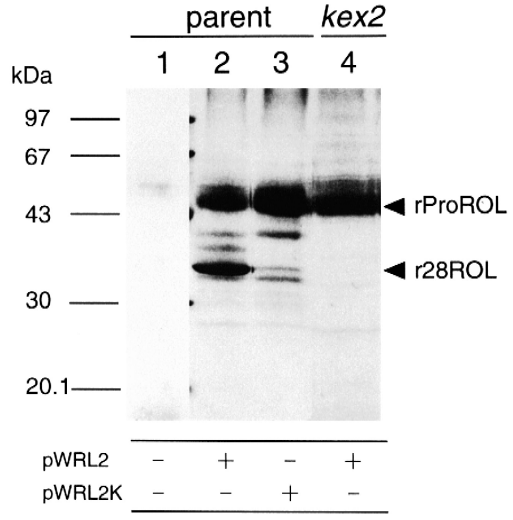

Fig. 2. Analysis of the culture supernatants of the transformants harboring pWRL2 or pWRL2K by SDS-PAGE. The proteins in the culture supernatants were precipitated by acetone. After being desalted, 30 μg proteins solubilized from the precipitates were loaded onto the gel. Lanes: (1) MT8-1 (parent strain) harboring pMW1 (a control plasmid); (2) MT8-1 harboring pWRL2; (3) MT8-1 harboring pWRL2K; (4) KDH3 (*kex2* strain) harboring pWRL2.

disappeared, and only rProROL (46 kDa protein) was observed (Fig. 2, lane 4). These results strongly indicated that Kex2p was responsible for the cleavage. Furthermore, to demonstrate whether Lys(-30)–Arg(-29) was the recognition sequence of Kex2p, the plasmid pWRL2K harboring the mutated *ROL* gene was expressed in the host MT8-1 cells. The major protein secreted was rProROL, although a small amount of r28ROL was observed (Fig. 2, lane 3). These results demonstrated that rProROL was cleaved by Kex2p to give r28ROL, and that Lys(-30)–Arg(-29) in the prosequence of rProROL was the recognition sequence.

3.3. Co-expression of the ROL gene with the Kex2p-encoding gene

In order to produce only r28ROL, the *ROL* gene was co-expressed with the *KEX2* gene. The plasmid pWRL2 for expression of the *ROL* gene and the plasmid pUmKX for expression of

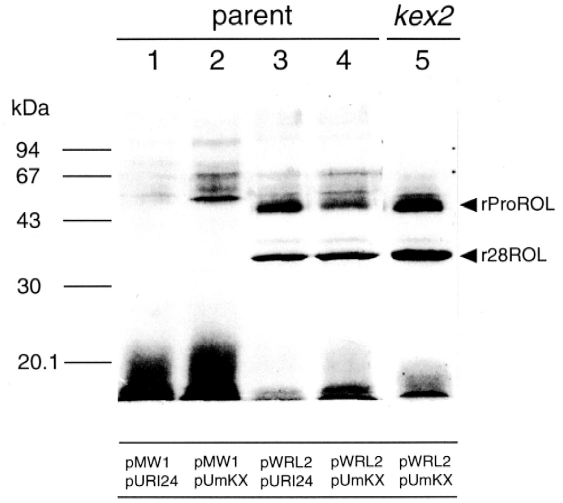

Fig. 3. Analysis of the culture supernatants of the transformants co-expressed *ROL* gene and *KEX2* gene by SDS-PAGE. The proteins in the culture supernatants were precipitated by acetone. After being desalted, 15 μg proteins solubilized from the precipitates were loaded onto the gel. Lanes: (1) MT8-1 (parent strain) harboring pMW1 and pURI24; (2) MT8-1 harboring pMW1 and pUmKX; (3) MT8-1 harboring pWRL2 and pURI24; (4) MT8-1 harboring pWRL2 and pUmKX; (5) KDH3 (*kex2* strain) harboring pWRL2 and pUmKX.

the *KEX2* gene were co-transformed into the MT8-1 cell, and the secreted proteins in the culture supernatant were analyzed by SDS-PAGE. In comparison with lanes 3 and 4 in Fig. 3, the amount of secreted rProROL decreased in the cells harboring the *KEX2* gene expression plasmid, pUmKX. The cleavage of rProROL was not complete. To confirm the activity of Kex2p derived from the expression plasmid, the plasmids pUmKX and pWRL2 were co-transformed to the *kex2* strain, KDH3, and the secreted proteins were analyzed. Although r28ROL was not observed in the culture supernatant of the KDH3 cells harboring pWRL2 (Fig. 2, lane 4), both r28ROL and rProROL appeared in the KDH3 cells harboring pWRL2 and pUmKX (Fig. 3, lane 5), i.e., rProROL was cleaved by Kex2p derived from the plasmid pUmKX. These results indicated that the active Kex2p was surely produced in the transformant cells but that there would be a limitation in its production in cells.

3.4. Co-expression of the ROL gene with the sKex2p-encoding gene

As described above, the cleavage of rProROL by Kex2p was not completed even when Kex2p was produced using the plasmid. To completely cleave rProROL to r28ROL, rProROL-encoding gene was co-expressed with the sKex2p-encoding gene, the product being secreted due to the deletion of the C-terminal region for anchoring in the Golgi membrane [21]. pWRL2 and pUsKX containing the sKex2p-encoding gene were co-transformed to the MT8-1 cell, and the cells were cultivated in YPDBC medium because sKex2p is unstable at either acidic or basic condition and requires a calcium ion for its activity [12]. On the analysis by SDS-PAGE of the secreted proteins from the cells harboring both pWRL2 and pUsKX, rProROL (46 kDa) was proved to disappear completely, and the r28ROL (35 kDa) and a 67 kDa protein were observed (Fig. 4, lane 3), while the rProROL was detected in proteins secreted from the cells harboring both pWRL2 and pURI24, a control

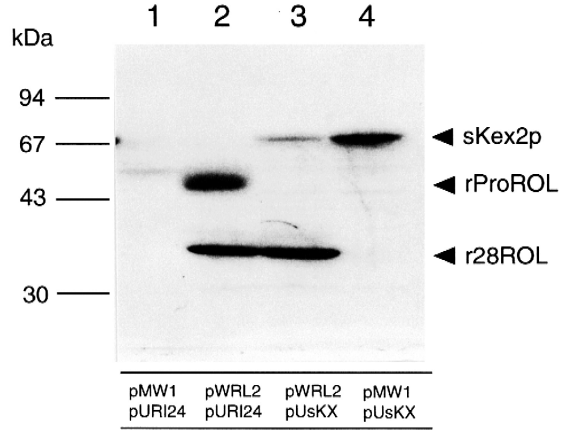

Fig. 4. Analysis of the culture supernatants of the transformants co-expressed *ROL* gene and *KEX2Δ613* gene by SDS-PAGE. The proteins in the culture supernatants were precipitated by acetone. After being desalted, 10 μg proteins solubilized from the precipitates were loaded onto the gel. Lanes: (1) MT8-1 harboring pMW1 and pURI24; (2) MT8-1 harboring pWRL2 and pURI24; (3) MT8-1 harboring pWRL2 and pUsKX; (4) MT8-1 harboring pMW1 and pUsKX.

plasmid (Fig. 4, lane 2). The 67 kDa protein was confirmed to be sKex2p because the molecular mass of the secreted protein from the cells harboring pUsKX and pMW1, a control plasmid, was about 67 kDa on SDS-PAGE (Fig. 4, lane 4), and the N-terminal amino acid sequence was identical to that of the mature form of Kex2p [12]. These results showed that the active sKex2p was co-produced with the target protein and that the processing of rProROL was completely performed by the co-produced sKex2p (Fig. 3, lane 4 and Fig. 4, lane 3).

4. Discussion

Here we reported the complete processing of rProROL by co-production of the soluble form of Kex2p (sKex2p) in *S. cerevisiae*. In the secretion of heterologous proteins from *S. cerevisiae*, a prepro-α-factor leader sequence has been often used [16] because it is usually cleaved and removed by Kex2p in the late Golgi compartment. However, recombinant proteins still possessing the propeptide of α-factor are often obtained [11,16], and mixtures of completely and incompletely cleaved forms disturb the purification. To get the completely processed proteins, construction of a system, in which complete cleavage by Kex2p can be performed, is essential.

In yeast, Kex2p, Yap3p, and Mkc7p have been reported as endoproteases which cleave the proteins at dibasic amino acids [6,26,27]. However, the protein secreted from the *kex2* cells was only rProROL (Fig. 2, lane 4), 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 [27–29].

A complete processing of a protein by overexpression of the Kex2p-encoding gene has already been reported [30], while in our experiments, the co-production of Kex2p was not effective for the complete cleavage of rProROL. This discrepancy may occur due to the difference in the amounts of secreted proteins or the difference in the three-dimensional structures around the cleavage sites because it would be difficult for Kex2p to approach the sites sterically hindered. Limitation of the amount of Kex2p on intracellular expression was also reported by Seeboth and Hein [14]. The degradation rate of Kex2p and the capacity of Kex2p in the Golgi membrane would also be taken into consideration.

Kex2p secreted by deleting the C-terminal amino acids for membrane anchoring [12,21] was used in vitro for cleaving α-factor leader fusion proteins and His tag fusion proteins bearing the Kex2p cleaving site [14,15]. Unlike such experiments in vitro, our results showed that 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 proteins 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 being secreted into the culture medium.

The cells harboring the *KEX2Δ613* gene lacking the 603 bp fragment encoding the C-terminal region including the TMD will be useful for the production of foreign proteins necessary for cleavage at the internal Lys–Arg site for maturation.

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