Secretion and Overproduction of Carboxypeptidase Y by a *Saccharomyces cerevisiae* ssll Mutant Strain

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Carboxypeptidase Y (CPY; EC 3.4.16.1) is the yeast vacuolar protease. To have CPY secreted and to increase its secretion level, we tried to express the prepro-CPY gene under the control of the inducible GAL10 promoter or constitutive ENO1 promoter on a multicopy plasmid. In the strains KK4, PEP4, and A2-1-1A, carrying the CPY expression plasmid, active CPY was not detected in the culture broth although the CPY activity was greatly increased inside the cells. In contrast, when we used a strain that contained the ssll (super-secretion of lysozyme) mutation, a large amount of active CPY (about 10–50 mg/liter) was detected in the culture broth. The ssll mutants secreted active CPY when the CPY level was increased by expressing it under the control of a strong promoter on a multicopy plasmid, while the endogenous expression of chromosomal CPY gene in the same ssll mutant caused a deficiency in the processing of pro-CPY to mature CPY.

Carboxypeptidase Y (CPY) is a glycoprotein (61 kDa) located in the vacuoles of the yeast *Saccharomyces cerevisiae*. CPY is synthesized as a 67-kDa inactive precursor (prepro-CPY) that is translocated into the lumen of the endoplasmic reticulum to form pre-CPY, which receives N-glycosidically linked core oligosaccharides. After further glycosyl modifications pre-pro-CPY is targeted to the vacuoles, where pre-CPY is processed into the mature form (61 kDa) by cleavage of a part of the N-terminal pro-region. This maturation of CPY is dependent upon the presence of protease ysc A and protease ysc B. CPY is a serine carboxypeptidase. However, in addition to peptidase activity, CPY has the ability to catalyse aminolysis of *C*-terminal peptide esters *in vitro*, resulting in amidation of the peptide (H. Tamaoki, unpublished data).

Nielsen et al. have already succeeded in causing the secretion of about 40 mg of CPY per liter of culture broth (this level represents a 200-fold overproduction as compared to a ypll strain with a single copy of the chromosomal CPY structural gene) when the CPY structural gene was placed under the control of the GAL10 promoter on a high copy number plasmid in a ypll mutant. They reported that high level production of CPY resulted in two molecular weight forms (56, 61 kDa) of active extracellular CPY, probably with different carbohydrate contents.

To achieve the overproduction and secretion of this enzyme into the media, we intended the expression of the prepro-CPY structural gene cloned from an *S. cerevisiae* gene library under the control of an inducible GAL10 promoter or constitutive ENO1 promoter on a multicopy plasmid in *S. cerevisiae*. In this report, we describe how the expression of the prepro-CPY structural gene, which is placed either under the inducible GAL10 promoter or the non-inducible ENO1 promoter on a micronutrient based plasmid, causes the overproduction of CPY leading to the secretion of a large fraction of newly synthesized enzyme into the culture medium in the ssll mutant. The extracellular CPY thus obtained from the culture broth was an active mature form, while CPY expressed from a single copy of chromosomal CPY gene was mostly an intra-cellular inactive precursor form in the same strains.

**Materials and Methods**

**Strain and plasmids.** *Saccharomyces cerevisiae* strains and plasmids used in this study were KS58-2D (a, ssl1, leu2, his3, ura3), KSM169 (a, ssl1, leu2, his3 or his3, ura3), KK4 (a, leu2, ura3, trpl, his3 or his3, gal80), A2-1-1A (a, Suc2, mal, CUP1, gal2, leu2), PEP4 (a, leu2, pep4), and plasmids YEp-HLYS1G and pEsh. The cloning of the CPY structural gene was done by the colony hybridization method. Yeast chromosomal DNA was isolated from *S. cerevisiae* SANK 50182, which is sold commercially as baker's yeast by Sankyo Co., Ltd. A yeast genomic library was constructed by inserting the HindIII DNA fragments of yeast chromosomal DNA in the HindIII site of pUC19. The yeast genomic library was screened with a 32P-labelled CPY coding region probe (5'-TGTTCCAGCTACCATTTA-3'). Twenty-two positive colonies were detected from about 32,000 *E. coli* transformants. The corresponding to the CPY structural gene cloned was identical with the DNA sequence described by Valls et al. We constructed a multicopy plasmid, pCY303 (Fig. 1), which contains the CPY structural gene with the prepro-CPY region inserted between the yeast GAL10 promoter and the 2p plasmid FLP terminator in the *S. cerevisiae*- *E. coli* shuttle vector plasmid YeplHyLS1G with a LEU2 selection marker. The pCY401 plasmid was constructed by replacing the BamHI–SalI fragment carrying the GAL10 promoter region (500 bp) in pCY303 plasmid with the BamHI–SalI fragment carrying the ENO1 promoter region (742 bp) in pEsh plasmid (Fig. 1).  

**Media and cultural conditions.** The *E. coli* cells were grown in LB-broth (1% Bacto trypton (Difco, U.S.A.), 0.5% yeast extract (Difco, U.S.A.), 0.5% NaCl, and 0.2% glucose). Ampicillin was added where appropriate to a final concentration of 80 µg/ml. The *S. cerevisiae* cells were grown in YPD medium (2% Bacto trypton, 1% yeast extract, and 2% glucose). In the experiment to express the CPY genes, SD medium was used containing 0.67% Bacto yeast nitrogen base without amino acids (Difco), with an amino acid mixture (20–375 µg/ml) lacking leucine, 2% glucose, 

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**Abbreviations:** CPY, carboxypeptidase Y; ssll, super-secretion of lysozyme.
Fig. 1. Construction of CPY Expression Plasmids Using the GAL10 Promoter (pCY303) and the ENO1 Promoter (pCY401).

The pCY101 plasmid was constructed by introducing a CPY gene isolated from chromosomal DNA of S. cerevisiae SANK50182 into the HindIII site of pUC19. The AccI fragment carrying pre-pro CPY gene was excised from pCY101 plasmid. The AccI site of this fragment was changed to a Sall site by linker insertion. The 2200-bp fragment containing pre-pro CPY gene was excised from pCY201 by SalI and HindIII digestion and ligated into the large Sall–HindIII fragment of YEp-HLYSIG. The resultant plasmid, pCY303, has the CPY structural gene with the pre-pro CPY region inserted between the yeast GAL10 promoter and FLP terminator, yeast 2μ plasmid containing a replication origin (ori), yeast DNA containing LEU2 gene, and E. coli DNA containing a replication origin (ori) derived from pBR322. Similarly, the 2200-bp fragment containing the pre-pro CPY gene excised from pCY201 was ligated into the large Sall–HindIII fragment of pESH. The resultant plasmid, pCY401, has the same DNA region as pCY303 except for replace went of the GAL10 promoter to the ENO1 promoter.

0.2M potassium phosphate buffer (pH 7.6), and 0.5% bovine serum albumin (for a stabilizer). Two percent galactose was added to SD medium for the induction of the GAL10 promoter. S. cerevisiae cells harboring pCY303 or pCY401 plasmids were grown initially in SD medium, with or without 2% galactose, respectively, for 3 days at 26°C. After inoculation with 1 ml of seed culture, the cells were cultivated in 100 ml of SD medium, with or without 2% galactose, for 7 days at 28°C on a rotary shaker (200 rpm), and the course of the secretion of CPY was monitored by measuring the CPY activity.

Measurement of enzymatic assay. The CPY activity was measured photometrically as follows. An assay sample (10 μl) was added to 1 ml of 1 mM CBZ-Phe-Leu solution in 0.1M Na–phosphate buffer (pH 7.0). The initial decrease in the absorbance at 224 nm was measured at room temperature for 5 min. One unit corresponds to 7 μg of CPY under these assay conditions.

Preparation of cell extract. Cell extract was prepared to measure intracellular enzymatic activities and to perform Western blot analysis as follows. Cells were washed with water and then with 100 mM Tris–HCl (pH 7.5). The cells resuspended in the above buffer were disrupted by a Braun cell homogenizer (Kaiser) for 3 min in the presence of glass beads.
(0.45–0.55 mm (8)). The suspension was centrifuged at 15 krpm for 10 min at 4°C. The supernatant fraction was used for intracellular enzymatic assays and Western blot analysis.

**Western blot analysis.** Intracellular and extracellular samples were put onto a SDS-polyacrylamide (9%) slab gel and electrophoresed. The separated proteins were electrophoretically transferred to PVDF membranes for protein-blotting-based applications (Millipore) and developed with CPY antibody and horseradish peroxidase-conjugated goat anti (rabbit IgG) antibody, following the manufacturer’s instructions.

**Results**

**High level secretion of CPY under the control of regulated yeast GAL10 promoter**

To obtain secretion of overproduced CPY, we constructed the 2μ-based plasmid pCY303 (Fig. 1) containing the CPY structural gene with the pre-pro CPY region under the control of the S. cerevisiae inducible GAL10 promoter. When grown on SD medium lacking leucine, supplemented with 2% galactose for 3 days, KSS8-2D/pCY303 and KSM-169/pCY303 produced 10.3 mg/liter and 11.1 mg/liter enzymatically active extracellular CPY, and only 0.4 mg/liter and 0.09 mg/liter active intracellular CPY, respectively (Table). Thus the increased gene dosage of the CPY structural gene under the inducible GAL10 promoter resulted in 180-fold overproduction of active extracellular CPY, as compared to a single copy CPY gene in the strain KS8-2D or KSM-169 without plasmids (0.06 ml/liter). In contrast, as shown in Table, when the strains KK4, A2-1-1A, and PEP4 carrying a multicopy plasmid pCY303 were cultivated in the same SD medium with 2% galactose, extracellular CPY was not detected while the increases in the amount of the intracellular CPY were observed except for strain KK4. These results indicate that ssl (superscretion of lysozyme) mutant strains (KS8-2D or KSM-169) have a higher ability to secrete overproduced CPY. The amount of secreted CPY by the strain KS8-2D/pCY303, linearly increased until 90 h of cultivation but decreased after 90 h of cultivation although the amount of intracellular CPY slowly increased until the end of cultivation (125 h), as shown in Fig. 2. Production of secreted active CPY corresponds to 90% of the total active CPY in 92-h cultivated cells (Fig. 2).

In the strain KK4, the intracellular CPY was detected as an inactive precursor form (pro-CPY) on Coomassie-blue-stained SDS-polyacrylamide gels although that corresponded with an active mature form CPY in the strain A2-1-1A and PEP4 (data not shown). In PEP4 cells containing a pep4 mutation, which causes a reduced activity of vacuolar proteinase A, B, and CPY, a high activity of CPY was detected inside the cell by the CPY overproduction (Table). Accordingly, it is likely that the large amount of overproduced CPY may be localized not only in the vacuole but also in the periplasmic space, as described in vpll strain by Stevens et al.11) and the conversion of pro-CPY to active CPY may be mediated not only by a PEP4-dependent manner in the vacuole but also by a PEP4-independent manner in the periplasmic space. Furthermore, the lower CPY activity inside the cell in KK4 cells than in A2-1-1A or PEP4 cells suggests that the conversion level of pro-CPY to active CPY is lower in KK4 cells than in A2-1-1A or PEP4 cells.

**Fig. 2. Courses of Intracellular and Extracellular CPY Production by ssl Mutant Strain (KSS8-2D) Carrying pCY303.**

After S. cerevisiae strain KSS8-2D harboring pCY303 was grown in SD medium containing 2% glucose and 2% galactose with 0.5% BSA for 3 days at 26°C two times, the culture was diluted in a 1:99 ratio with fresh SD medium with 2% glucose, 2% galactose, and 0.5% BSA. The cells were cultivated in 100 ml of SD medium at 28°C with shaking. A sample (10 ml) of cells was taken at each time indicated and the supernatants and the cells were obtained by centrifugation. The courses of intracellular and extracellular CPY production were monitored by measuring the CPY activity as described in Materials and Methods. The amount of CPY is shown as extracellular CPY (●) and intracellular CPY (○).

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<td>Extracellular</td>
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<td>Strain</td>
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<td>KSS8-2D</td>
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<td>KSM-169</td>
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<td>KK4</td>
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<td>PEP4</td>
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Yeast cells harboring the plasmid pCY303 or without plasmid were grown in SD medium containing 2% galactose at 28°C for 3 days on a rotary shaker (200 rpm). The amount of CPY was calculated according to the standard activity curve obtained by authentic carboxypeptidase Y (Sigma). The values are the means of two independent experiments. N.D. = not detected.

**Increase in the stability of secreted CPY by BSA in the medium**

We added bovine serum albumin (BSA) as a carrier protein to the culture medium to protect secreted CPY against extracellular protease as described in strain vpl1.11) As shown in Fig. 3, the amount of active extracellular CPY using the strain KSS8-2D/pCY303 was greatly increased by growing the cells in SD medium with 0.5% BSA as compared to SD medium without BSA. In addition, we observed that the amount of band corresponding to BSA on Coomassie-blue-stained SDS-polyacrylamide gel decreased during the cultivation (data not shown). The stability of secreted CPY may be promoted in the BSA additional culture medium.

**Western blotting analysis of intracellular and extracellular CPY by ssl mutant strain**

We have already reported that the ssl mutant strains
Alteration in Pro-CPY Processing by CPY Overproduction

Fig. 3. Increase in the Stability of Secreted CPY caused by Adding BSA to Media.
The strain KS58-2D carrying pCY303 plasmid was grown in SD medium with (●) or without (○) 0.5% BSA at 28°C after the cells were cultivated in SD medium at 26°C two times as described in Fig. 2. The course of the secretion of CPY was monitored by measuring the CPY activity of supernatants obtained by centrifugation.

Fig. 4. Western Blotting Analysis of Intracellular and Extracellular CPY by the Strain KS58-2D.
*S. cerevisiae* strain KS58-2D with or without the plasmid pCY303 was grown in SD medium containing 2% galactose with 0.5% BSA for 3 days at 28°C after the cells were cultivated at 26°C two times, as described in Fig. 2. The amount of each cell fraction analyzed corresponds to 10 μl of the culture broth. The positions of migration of pro-CPY and mature CPY are noted.

have a lower CPY activity due to a deficiency in conversion of proenzyme to mature enzyme.6) It was also found that the proenzyme was not in the vacuole but in the periplasmic space in this strain, suggesting the mislocalization of vacuolar CPY in ssiI mutant (K. Suzuki, C. Oka, K. Ichikawa, and Y. Jigami, unpublished data). However, we showed that the ssiI mutant secreted a large amount of active CPY in the culture medium when the CPY structural gene was overexpressed under the control of GAI10 promoter on a multi-copy plasmid in the ssiI mutant strains. Then, intracellular and extracellular CPY synthesized in the strain KS58-2D/pCY303 were analysed by Western blotting. As presented in Fig. 4, a large amount of extracellular CPY was detected as an inactive mature form CPY, while intracellular CPY was detected as a mixture of pro-CPY and mature CPY, suggesting that secreted CPY was converted from inactive pro-CPY to active mature CPY when the CPY level in the cell was increased by expressing the CPY gene under the control of a strong promoter on a high copy number plasmid.

Fig. 5. Courses of Cell Growth and Amount of Secreted CPY by the Strain KS58-2D Carrying pCY401 Plasmid.
Under the condition described in Fig. 2, the strain KS58-2D carrying pCY401 plasmid was grown in SD medium containing 2% glucose and 0.5% BSA. The amount of secreted CPY was monitored by measuring the CPY activity at each time indicated.

**ENO1 promoter directed CPY overproduction**

The GAI10 promoter is inducible by galactose. Nielsen *et al.* described how the stability of plasmid containing CPY gene and growth rates of yeast cells were reduced by the overexpression of CPY gene under the GAI10 promoter in the logarithmic phase.5) Then, they repressed the expression of CPY gene during the logarithmic phase by the presence of glucose and induced it in the stationary phase by the glucose consumption and galactose use as a carbon source. To compare the CPY expression level under the different promoters, the original GAI10 promoter was replaced with the ENO1 promoter, the expression of which is not repressed during cell growth on a glucose medium. The ENO1 promoter encodes enolase, one of the glycolytic enzymes and directs a high level of transcription in cells grown on glucose.

Figure 5 shows the cell growth and the amount of CPY secreted into the culture medium by the strain KS58-2D carrying the plasmid pCY401 grown on 2% glucose containing 0.5% BSA. The level of secreted CPY under the ENO1 promoter was as high as that under the GAI10 promoter, suggesting that the CPY gene can be overexpressed not only under an inducible promoter but also under a constitutive promoter by the strain KS58-2D.

**Discussion**

Nielsen *et al.*5) achieved growth-phase-dependent over-
production of secreted CPY in vplI strain due to the repression of GALI promoter-directed CPY gene expression by glucose during exponential growth and the subsequent induction of it by galactose when the growth rate decreases. At first, we expressed the CPY gene under the control of inducible GALI0 promoter in the strain KS58-2D/pCY303 grown on SD medium with a mixed carbon source (2% glucose and 2% galactose) and succeeded in obtaining about 10–50 mg of active CPY per liter of culture medium (180-fold overproduction as compared to the strain KS58-2D with a single copy of the CPY gene). Next, to get efficient CPY production during exponential cell growth, we tried to replace the original GALI0 promoter with the constitutive ENO1 promoter. The CPY secretion level in the strain KS58-2D/pCY401 (control of ENO1 promoter) grown on SD medium with 2% glucose reached almost the same level as that in the strain KS58-2D/pCY303 (control of GALI0 promoter) grown on SD medium with 2% glucose and 2% galactose. In addition, the growth rate in KS58-2D/pCY401 was rather faster than that in KS58-2D/pCY303. These results appear to suggest that growth rates and stability of plasmid in the strain KS58-2D which we used may be not influenced by the expression of the CPY gene during exponential growth.

Nielsen et al.5) have described how in vpl strains the protease-catalyzed maturation of pro-CPY was inhibited by adding BSA as a carrier protein in the medium. However, we showed that in ssli strains the amount of active CPY in the medium was increased rather than by addition of BSA to the medium than by no addition of BSA (Fig. 3). Presumably, this difference may reflect some difference in the maturation enzyme of pro-CPY in ssli strains which we used from that in vplI strains or it may be in a different place.

In ssli strains carrying a single copy of the wild-type CPY gene, we have already shown that the ssli mutant strain accumulate pro-CPY inside the cells,6) probably due to the mislocalization of vacuolar CPY to the periplasmic space (K. Suzuki, C. Oka, K. Ichikawa, and Y. Jigami, unpublished data). But in this paper, we described how the maturation of pro-CPY may be greatly promoted by overproduction of CPY. It is not clear why overproduction of CPY has this positive effect on the maturation of pro-CPY. We assumed that the increase of CPY maturation may be caused by the following two putative hypo-

1. The amount of both mature CPY and pro-CPY secreted was increased by expressing CPY gene under the control of strong promoter on the multicopy plasmid. An unknown protease secreted in the medium may prefer pro-CPY to mature CPY as the substrate, because it is assumed that pro-CPY cannot make a compact conformation compared with mature CPY. Thus, most of pro-CPY secreted in the medium was degraded rapidly by this unknown protease and only mature CPY remained in the medium.

2. The activity of an unknown CPY maturation enzyme, which is in the periplasmic space or medium, may be enhanced by some vacular enzymes. And this vacular enzyme may be mislocalized to the periplasmic space or the medium because the transport of that to vacuole was disturbed by CPY overproduction. Then, the activity of the unknown CPY maturation enzyme may be increased by the mislocalization of this vacular enzyme, and a large amount of mature CPY may be secreted in the medium.

At present, it is not clear whether the processing site of pro-CPY to form active CPY in ssli strain is identical with that observed in vacuoles in the wild type cells. Further biochemical analysis is necessary to elucidate the mechanism by which the maturation of pro-CPY is promoted by overproduction of CPY.

References