J. Microbiol. Biotechnol. (2005), **15**(1), 202–205

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of Active Carboxypeptidase Y of *Saccharomyces cerevisiae* Secreted from Methylotrophic Yeast *Pichia pastoris*

RO, HYEON-SU, MI-SUN LEE, MOON-SUN HAHM, HEE-SUNG BAE, AND BONG HYUN CHUNG*

BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yuseong, Daejeon 305-600, Korea

Received: March 12, 2004
Accepted: April 27, 2004

Abstract Our previous study showed that the overexpression of carboxypeptidase Y (CPY) of *Saccharomyces cerevisiae* in *Escherichia coli* resulted in the formation of insoluble inclusion bodies. To produce soluble CPY, we designed a novel *Pichia pastoris* expression system, in which the following were inserted into expression vectors: three different signal sequences derived from the mating factor a1 of *S. cerevisiae*, an inulinase of *Kluyveromyces marxianus*, and the endogenous signal sequence of CPY. The expression vector pHIL-D2-SS_{inul}-proCPY was the most effective in the production of proCPY among the vectors examined. The purified active CPY was obtained from proCPY by treating with proteinase K, followed by Q-Excellose ion-exchange column chromatography.

Key words: Carboxypeptidase, pichia expression, proteinase K, secretory

Carboxypeptidase Y (CPY) from *Saccharomyces cerevisiae* is a serine carboxypeptidase that catalyzes the removal of C-terminal amino acid from peptides and proteins. Earnest attempts have been made to produce CPY, because of its potential application [1, 3, 4, 8, 9]. One of the strategies to produce CPY was the use of mutant strains of *S. cerevisiae* that have defects in the vacuolar protein sorting [13] or in the secretion [10, 15]. We recently reported the production of CPY or proCPY in *Escherichia coli* [5–7]. However, most of the expressed CPY or proCPY was present in the form of inclusion bodies in cytoplasm. In general, the inclusion bodies are activated through *in vitro* denaturation and renaturation processes [17], however, a large fraction of CPY activity are lost during the processes. As a series of studies on the production of CPY, we designed a novel CPY expression system in a methylotrophic yeast, *Pichia pastoris*, which has frequently been chosen for the production of foreign proteins [12].

*Corresponding author
Phone: 82-42-860-4442; Fax: 82-42-879-8594;
E-mail: chungbh@kribb.re.kr

Construction of CPY Expression Vectors

The plasmids pPIC9 and pHILD2 (Invitrogen, CA, U.S.A.) were used as expression vectors for the production of CPY in *P. pastoris* (GS115, *his*4 strain, Invitrogen, CA, U.S.A.). Expression of the target protein is under the control of a strong *AOX1* promoter for both vectors. The pPIC9 vector is an expression vector that contains a signal sequence of α-mating factor (*MFα1*) for secretory expression of a target protein. *PRC1*, devoid of N-terminal 60 bps which corresponds to the signal peptide, was PCR-amplified from pESC-CPY as a template DNA [5], using the following pair of primers: N-primer, 5'-tct ctc aga aaa aga atc tca ttg caa aga ccg-3', and C-primer, 5'-ggc gaa ttc tta taa gga gaa acc acc gtg gat cc-3'. The PCR product was digested with *Xho*I and *Eco*RI and ligated with pPIC9 that was pre-digested with the same enzymes to yield pPIC9-$SS_{MF\alpha1}$-proCPY (Fig. 1). To construct a plasmid that contains the proCPY gene fused with an inulinase signal sequence in pHIL-D2 vector, the following procedure was adopted: First, a gene encoding the signal sequence of inulinase in *Kluyveromyces marxianus* was PCR-amplified, using an N-primer, 5'-gct tca gaa ttc atg aag tta gca tac tcc ctc ttg c-3', and a C-primer, 5'-gta att aat aac tga agc act gac tcc tgc caa-3', and the PCR product was digested with *Eco*RI (N-terminus) and *Asn*I (C-terminus). Second, the proCPY gene was PCR-amplified, using an N-primer, 5'-gct tca gtt att aat tac aag aga atc tca ttg caa aga ccg-3', and a C-primer, 5'-ggc gaa ttc tta taa gga gaa acc acc gtg gat cc-3', and the PCR product was digested with *Asn*I (N-terminus) and *Eco*RI (C-terminus) (Fig. 1). The resulting fragments were ligated into the *Eco*RI site of pHIL-D2 (pHIL-D2-SS_{inul}-proCPY). To construct pHIL-D2-SS_{endo}-proCPY that contained the endogenous *S. cerevisiae* proCPY signal sequence, the

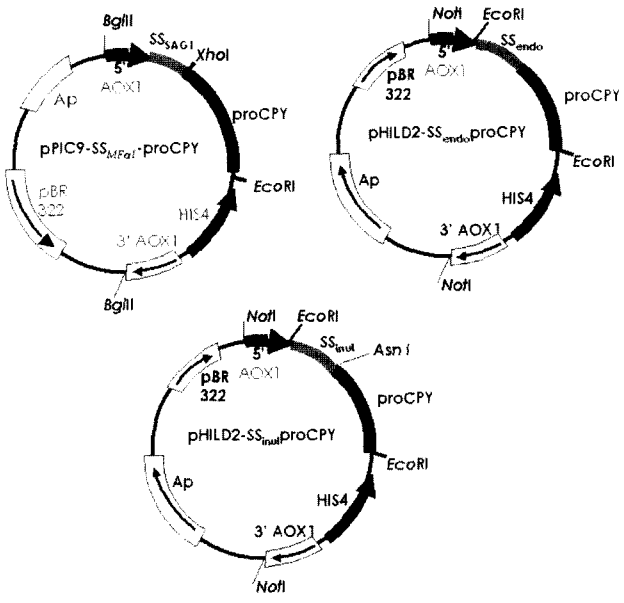

Fig. 1. Construction of proCPY expression vectors.
pPIC9-SS$_{MF\alpha 1}$-proCPY, pHIL-D2-SS$_{inul}$-proCPY, and pHIL-D2-SS$_{endo}$-proCPY contained a signal sequence from *S. cerevisiae MFα1*, a signal sequence of inulinase from *K. marxianus*, and an endogenous signal sequence of proCPY of *S. cerevisiae*, respectively.

whole *PRC1* gene was PCR-amplified with our previous construct as template DNA [5], using an N-primer, 5'-ggc gaa ttc atg aaa gca ttc acc agt tta cta tgt-3', and a C-primer, 5'-ggc gaa ttc tta taa gga gaa acc acc gtg gat cc-3'. The PCR product was digested with *Eco*RI and ligated into the *Eco*RI site of pHIL-D2 (Fig. 1). All the clones were confirmed by DNA sequencing. Transformation of the vectors to *P. pastoris* host was performed as recommended (Invitrogen, MA, U.S.A.)

Secretory Expression of ProCPY

The recombinant *P. pastoris* strain carrying an expression vector was grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Cells were harvested when the optical density of the culture at 600 nm reached 5. The cells harvested were resuspended in an induction medium [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 0.5% methanol] and cultured for an additional 48 h at 30°C. The prolonged culture was supplemented with an additional 1% methanol after 24 h.

The secretory expression of proCPY from *P. pastoris* was examined using three expression vector constructs, each of which contained different signal sequences. When proCPY was expressed using the expression vector pPIC9-SS$_{MF\alpha 1}$-proCPY that contained a signal sequence from the *S. cerevisiae* α-mating factor, the level of the proCPY expression was quite poor. Either in the intracellular fractions or in the extracellular fractions, no major protein band corresponding to proCPY was detected on Coomassie-stained protein gel (Figs. 2A, 2B). The expression was detectable only by immunoblot analysis with prolonged exposure time. To improve the expression level, we inserted a whole *PRC1* gene, including the endogenous *S. cerevisiae* CPY signal sequence, into a pHIL-D2 plasmid, nevertheless, the expression was still not detectable (data not shown). Finally, we fused the inulinase signal sequence from *K. marxianus*, which had frequently been chosen for the secretory expression of proteins in yeast [11], to the gene encoding proCPY, thus generating pHIL-D2-SS$_{inul}$-proCPY (Fig. 1). With the pHIL-D2-SS$_{inul}$-proCPY expression vector, a significant amount of proCPY was detected within 24 h of cultivation. After 48 h, proCPY was detected as one of the major bands on Coomassie-stained protein gel with both intracellular and extracellular fractions. Immunoblot

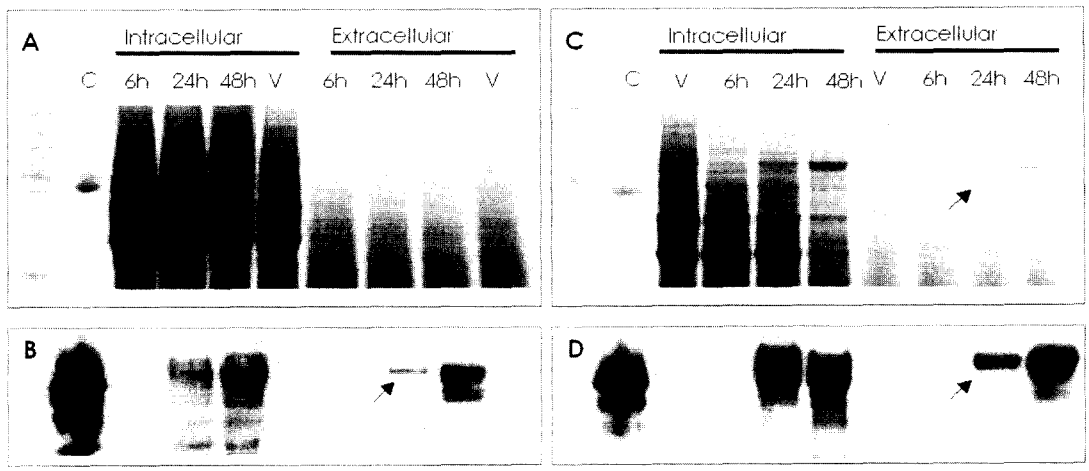

Fig. 2. Expression of proCPY from pPIC9-SS$_{MF\alpha 1}$-proCPY (Panels A and B) and pHIL-D2-SS$_{inul}$-proCPY (Panels C and D).
Lane C: 2 μg of commercial CPY; Lane V: expression pattern with empty vector; Lane 6 h, 24 h, and 48 h: expression patterns of proCPY after induction at given times. Panel A and panel C are SDS-PAGE analyses of the expression of proCPY. Panel B and panel D are immunoblot analyses of the gel from A and C with an anti-CPY antibody. The blotted membrane was exposed for 5 min (panel B) and 3 sec (panel D). Arrows indicate proCPY.

analysis confirmed that the major band on the protein gel was indeed the proCPY protein (Figs. 2C, 2D).

The above results suggest that the signal sequences used for the construction of expression vectors affect the production and secretion of proCPY in *P. pastoris*. Among the signal sequences tested, the inulinase signal sequence resulted in the highest proCPY production, suggesting that this sequence would be the most accessible to the *P. pastoris* translocation system. Any *P. pastoris* carrying expression vectors did not produce mature CPY, suggesting either that *P. pastoris* may not have an endogenous PrA and/or PrB capable of cleaving the propeptide from proCPY or that it has a less efficient vacuolar processing system.

Preparation of Active CPY

The proCPY protein from the culture broth of *P. pastoris* carrying pHIL-D2-SS_{inul}-proCPY was subjected to purification. Thus, the broth was concentrated by ultrafiltration, and the concentrated suspension was treated with proteinase K (Sigma, MO, 1 mg/100 mg-proCPY) for 30 min at 37°C to remove the N-terminal propeptide. After proteinase K treatment, the reaction mixture was loaded onto an ion-exchange column. The column was eluted with 0-0.5 M NaCl gradient solution and the fraction containing an active CPY was obtained at 0.45 M NaCl concentration (Fig. 3, Lane 5). The purified CPY exhibited the same molecular mass as that of commercially available authentic CPY, indicating the presence of a similar amount of glycosylation (Fig. 3). That is, CPY expressed in *E. coli* exhibited significantly higher mobility on SDS-PAGE gel, due to lack of glycosylation, although this is not expected to affect the enzyme activity [6]. Samples at each purification step were analyzed, and the result is summarized in Fig. 3: From 28.8 mg of total proteins in 180 ml of the culture broth, 0.2 mg of active CPY was obtained, and percentages of purity of the final preparation and the yield were 60.3% and 4.2%, respectively.

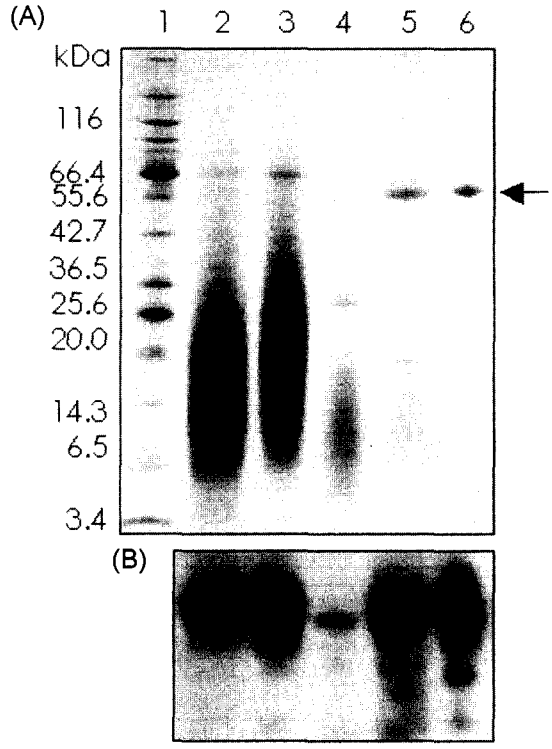

Fig. 3. SDS-PAGE and immunoblot analyses during the purification steps.
(A) SDS-PAGE analysis. Lane 1: molecular weight size marker; lane 2: the culture broth; lane 3: the broth concentrated by ultrafiltration; lane 4: the reaction mixture after proteinase K treatment; lane 5: the CPY purified with an anion-exchange column chromatography; lane 6: the commercially obtained CPY. Arrow indicates the mature CPY. (B) Immunoblot analysis of the gel from (A) with an anti-CPY antibody.

We compared the activity of the purified CPY with that of commercially available CPY on the hydrolysis of N-(2-furanacryloyl)-L-phenylalanyl-L-phenylalanine (FAPP) [6]. The purified enzyme showed exactly the same specific activity as that of the commercial CPY, whereas proCPY exhibited virtually no activity (data not shown). The presence of propeptide has been known to inhibit the CPY activity [16]. In *S. cerevisiae*, the propeptide is cleaved off by vacuolar protease PrA and/or PrB, and overexpression of proCPY in *S. cerevisiae* secretes mature CPY into culture broth [10, 13, 15]. On the contrary, however, our results indicated that *P. pastoris* could not cleave off the propeptide of CPY. A homology search of *P. pastoris*-related methylotrophic yeast found that *P. angusta* had both PrA and PrB homologues, suggesting the presence of PrA and PrB processing systems in the vacuole of *P. pastoris* [2]. Thus, our data imply that *P. pastoris* may have a less efficient vacuolar processing system, which processes the propeptide of vacuolar-targeted proteins. This implication is further supported by the fact that the overexpression of *P. pastoris* CPY under the control of *AOX1* promoter resulted in the secretion of proCPY, but not mature CPY and its intracellular accumulation [14]. Further studies on the vacuolar processing system in *P. pastoris* are needed to obtain more conclusive results.

Acknowledgments

This work was supported by a grant from the KRIBB Initiative Research Program and, in part, by the grant from Korea Science and Engineering Foundation through the Center for Advanced Bioseparation Technology (BSEP).

References

1. Berne, P., S. Blanquet, and J. Schmitter. 1992. Carboxypeptidase Y-catalyzed transpeptidation of esterified oligo- and

polypeptides and its use for the specific carboxyl-terminal labeling of proteins. *J. Am. Chem. Soc.* **114:** 2603-2610.
2. Blandin, G., L. Bertrand, A. Malpertuy, P. Wincker, F. François Artiguenave, and D. Dujon. 2000. Genomic exploration of the hemiascomycetous yeasts: 13 *Pichia angusta*. *FEBS Lett.* **48:** 76-81.
3. Breddam, K., F. Widmer, and M. Meldal. 1991. Amidation of growth hormone releasing factor (1-29) by serine carboxypeptidase catalysed transpeptidation. *Int. J. Peptide Protein Res.* **37:** 153-160.
4. Buckler, D. R., E. Haas, and H. A. Scheraga. 1993. C-Terminal labeling of ribonuclease A with an extrinsic fluorescent probe by carboxypeptidase Y-catalyzed transpeptidation in the presence of urea. *Anal. Biochem.* **209:** 20-31.
5. Hahm, M. S., J. H. Bae, E. S. Choi, and B. H. Chung. 1999. *In vitro* activation of yeast pro-carboxypeptidase Y expressed as inclusion bodies in *Escherichia coli*. *Biotechnol. Lett.* **21:** 881-885.
6. Hahm, M. S. and B. H. Chung. 2001. Refolding and purification of yeast carboxypeptidase Y expressed as inclusion bodies in *Escherichia coli*. *Protein Express. Purif.* **22:** 101-107.
7. Hahm, M. S. and B. H. Chung. 2001. *In vitro* formation of active carboxypeptidase Y from pro-carboxypeptidase Y inclusion bodies by fed-batch operation. *J. Microbiol. Biotechnol.* **11:** 887-889.
8. Henriksen, D. B., M. Rolland, M. H. Jakobsen, O. Buchardt, and K. Breddam. 1992. C-Terminal amidation of calcitonin by carboxypeptidase Y catalyzed transpeptidation with a photocleavable nucleophile. *Peptide Res.* **5:** 321-324.
9. Henriksen, D. B., K. Breddam, and O. Buchardt. 1993. Peptide amidation by enzymatic transacylation and photolysis. *Int. J. Peptide Protein Res.* **41:** 169-180.
10. Ichikawa, K., Y. Shiba, Y. Jigami, and N. Serizawa. 1993. Secretion and overproduction of carboxypeptidase Y by a *Saccharomyces cerevisiae ssl1* mutant strain. *Biosci. Biotechnol. Biochem.* **57:** 1686-1690.
11. Kang, H. A., S. W. Nam, K. S. Kwon, B. H. Chung, and M. H. Yu. 1996. High-level secretion of human alpha 1-antitrypsin from *Saccharomyces cerevisiae* using inulinase signal peptide sequence. *J. Biotechnol.* **48:** 15-24.
12. Kim, H. K., Y. K. Hong, H. E. Park, S. H. Hong, and Y. A. Joe. 2003. Secretory production of recombinant urokinase kringle domain in *Pichia pastoris*. *J. Microbiol. Biotechnol.* **13:** 591-597.
13. Nielsen, T. L., S. Holmberg, and J. G. L. Petersen. 1990. Regulated overproduction and secretion of yeast carboxypeptidase Y. *Appl. Microbiol. Biotechnol.* **33:** 307-312.
14. Ohi, H., W. Ohtani, N. Okazaki, N. Furuhata, and T. Ohmura. 1996. Cloning and characterization of the *Pichia pastoris PRC1* gene encoding carboxypeptidase Y. *Yeast* **12:** 31-40.
15. Shiba, Y., F. Fukui, K. Ichikawa, N. Serizawa, and H. Yoshikawa. 1998. Process development for high-level secretory production of carboxypeptidase Y by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **50:** 34-41.
16. Winther, J. R. and P. Sorensen. 1991. Propeptide of carboxypeptidase Y provides a chaeperone-like function as well as inhibition of the enzymatic activity. *Proc. Natl. Acad. Sci. USA* **88:** 9330-9334.
17. Zhao, Y., W. Ge, Y. Gong, and C. Zhang. 2003. Cloning, expression, and renaturation studies of reteplase. *J. Microbiol. Biotechnol.* **13:** 989-992.