J. Microbiol. Biotechnol. (2001), **11**(5), 887–889

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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In Vitro Formation of Active Carboxypeptidase Y from Pro-Carboxypeptidase Y Inclusion Bodies by Fed-Batch Operation

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Received: January 2, 2001
Accepted: July 4, 2001

Abstract The gene encoding yeast pro-carboxypeptidase Y (pro-CPY) has been cloned and expressed in *Escherichia coli*. Most of the expressed pro-CPY was accumulated as cytoplasmic insoluble aggregates. In our previous study [3], active CPY was obtained by renaturation of entirely denatured pro-CPY followed by *in vitro* proteolytic processing with proteinase K along with the activation process. The same refolding process was performed to produce an active CPY from pro-CPY inclusion bodies with renaturation buffers containing proteinase K at different concentrations. The refolding efficiency decreased from 25% to 2% in the renaturation buffers containing proteinase K at concentrations of 60 μg/ml and 0.6 μg/ml, respectively. In an attempt to increase the refolding efficiency with a lesser amount of proteinase K, a novel fed-batch refolding process was developed. In a fed-batch refolding, 99 ml of the renaturation buffer containing pro-CPY was gradually added into 1 ml of the renaturation buffer containing 60 μg/ml of proteinase K to give a final proteinase K concentration of 0.6 μg/ml. The fed-batch refolding process resulted in a refolding efficiency of 18%, which corresponded to a 9-fold increase over that (2%) in the batch process.

Key words: Pro-carboxypeptidase Y (pro-CPY), carboxypeptidase Y (CPY), refolding, proteinase K, *E. coli*

Carboxypeptidase Y (CPY) is a vacuolar protease, synthesized as a zymogen that is only rendered active upon proteolytic removal of the propeptide. CPY has been extensively studied as a model for eukaryotic intracellular protein transport, targeting, and maturation [6, 8]. CPY from *S. cerevisiae* is initially synthesized as an inactive prepro-enzyme with a signal peptide of 20 amino acids followed by a propeptide of 91 amino acids and a mature region of 421 amino acids. The 20-residue signal peptide directs translocation of prepro-CPY into the endoplasmic reticulum (ER). Upon translocation, the signal peptide is cleaved off, yielding the pro-CPY. In the lumen of the ER, the pro-CPY undergoes core glycosylation, and an additional glycosylation takes place during the transit through the Golgi complex. The propeptide serves as a signal for vacuolar targeting of the enzyme. In the vacuole, the propeptide is proteolytically removed, generating an active and mature CPY. In this process, the propeptide plays an important role in folding CPY into its final active configuration.

CPY has been widely used for determining C-terminal sequences of peptides and proteins. In addition, it has an ability to catalyze the aminolysis of C-terminal peptide esters *in vitro*, resulting in the elongation of peptide chains [10]. It has also been used for C-terminal amidation of peptides by transpeptidation reactions using amino acid amides as nucleophiles [1].

Due to the importance of CPY, several attempts have been made to overproduce CPY via recombinant DNA technology, mostly by using *S. cerevisiae* as an expression host [4, 9]. The yeast seems to be the best candidate as a recombinant host for the production of mature CPY from inactive pro-CPY, because *Escherichia coli*, the most widely used recombinant host, does not harness the capability for transforming pro-CPY into the mature CPY by *in vivo* proteolytic processing. More recently, however, we have successfully produced an active CPY from the pro-CPY inclusion bodies expressed in *E. coli* [3]. Two different pro-CPYs from *H. polymorpha* and *S. cerevisiae* were expressed as inclusion bodies in *E. coli*, and a refolding process was developed to obtain active CPYs from inclusion bodies. After conventional denaturation and renaturation steps, the renatured pro-CPY was digested *in vitro* by proteinase K to cleave off CPY propeptide, and then CPY was activated by CPY propeptide. In this process, however, the amount of proteinase K used was more than five times that of the pro-CPY. In the present

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study, therefore, a novel refolding process employing a fed-batch operation was developed to reduce the amount of proteinase K to be used for the proteolytic removal of CPY propeptide from pro-CPY.

The cDNA encoding pro-CPY was obtained by the PCR amplification from *S. cerevisiae* 2805 [2, 5] genomic DNA library. The plasmid pESC-CPY for the expression of pro-CPY in *Escherichia coli* has been described previously [3]. This expression system was placed under the control of the T7 lac promoter, and had a translational start codon inserted in the frame with the cDNA sequence encoding mature pro-CPY for the intracellular expression. The plasmid pESC-CPY was transformed into *E. coli* BL21(DE3). The transformants were grown to 0.6 OD_{600} at 37°C in shake flasks containing 100 ml of Luria-Bertani medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) with 100 µg/ml of ampicillin. The expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, MO, U.S.A.) to a final concentration of 1 mM, and the cells were grown for an additional 4 h at 37°C. The cells were harvested by centrifuging at 6,000 ×g for 10 min, resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 3 mM EDTA, and lysed by sonication. The soluble and insoluble fractions were then separated by centrifugation at 12,000 ×g for 10 min at 4°C.

The inclusion bodies were solubilized in 50 mM MES buffer (pH 6.5) containing 6 M guanidinium chloride (GdmCl). The solubilized proteins were incubated at 23°C for 30 min, and then rapidly diluted into 50 mM MES buffer (pH 6.5) containing 2 mM $CaCl_2$ to give a final GdmCl concentration of 0.1 M. Densitometric scanning showed that the pro-CPY concentration in the inclusion body preparation was 9.6 µg/ml, which accounted for 80% of the total protein (data not shown). After the diluted solution was renatured at 23°C for 1 h, proteinase K (Sigma) was added to a final concentration of 0.6, 6, or 60 µg/ml. The resulting solutions were further incubated for 1 h at 22°C, and then the activity of CPY in the renaturation buffer was measured at 37°C for a decrease in absorbance at 330 nm of 0.2 mM *N*-(2-Furanacryloyl)-L-phenylalanyl-L-phenylalanine (FAPP) (Sigma) in 50 mM MES buffer (pH 6.0) containing 1 mM EDTA, as described by Peterson *et al.* [7]. The refolding efficiency was estimated from the specific FAPP hydrolysis activity of the refolded CPY relative to that of the native *S. cerevisiae* CPY (sequencing grade, Sigma).

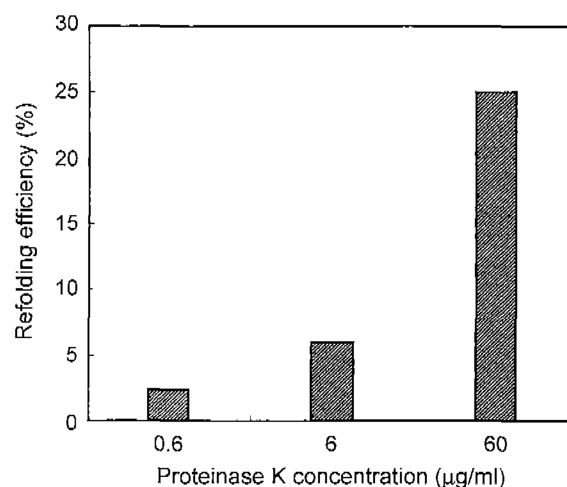

Fig. 1. Effect of proteinase K concentration on the refolding efficiency in the batch refolding process.
The inclusion bodies solubilized in 50 mM MES buffer (pH 6.5) containing 6 M guanidinium chloride (GdmCl) were rapidly diluted into 50 mM MES buffer (pH 6.5) containing 2 mM $CaCl_2$ to give a final GdmCl concentration of 0.1 M. After the diluted solution was renatured at 23°C for 1 h, proteinase K (Sigma) was added to a final concentration of 0.6, 6, or 60 µg/ml. The resulting solutions were further incubated for 1 h at 22°C, and then the activity of CPY in the renaturation buffer was measured at 37°C.

Figure 1 shows the effect of proteinase K concentration on the refolding efficiency. The refolding efficiency decreased from 25% to 2% in the renaturation buffers containing proteinase K at concentrations of 60 µg/ml and 0.6 µg/ml, respectively. This remarkable decrease in the refolding efficiency at 0.6 µg/ml of proteinase K appears to be attributed to an incomplete cleavage of CPY propeptide. Therefore, a major disadvantage of this refolding method is that it seems to require an excessive amount of proteinase

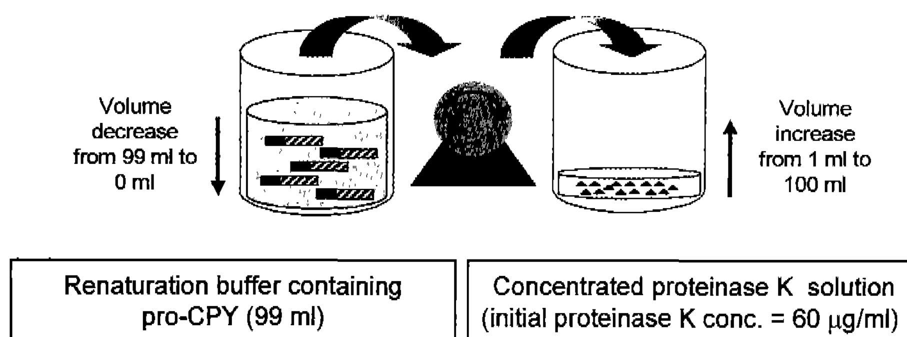

Fig. 2. Schematic description of the novel fed-batch refolding process for the production of active CPY from pro-CPY inclusion bodies.

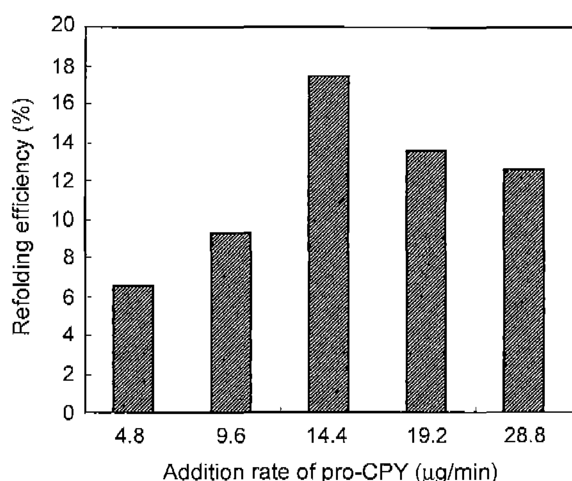

Fig. 3. Effect of pro-CPY addition rate on the refolding efficiency in the fed-batch refolding process.

The renaturation buffer (99 ml) containing pro-CPY was gradually added into 1 ml of the renaturation buffer containing 60 μg/ml of proteinase K to give a final proteinase K concentration of 0.6 μg/ml. After the feeding was complete, the CPY activity was measured.

K to obtain a substantial level of refolding efficiency. Unfortunately, therefore, this refolding process is not economically feasible.

A novel fed-batch refolding process was developed to increase the refolding efficiency with a lesser amount of proteinase K. In a fed-batch refolding, 99 ml of the renaturation buffer containing pro-CPY was gradually added into 1 ml of the renaturation buffer containing 60 μg/ml of proteinase K to give a final proteinase K concentration of 0.6 μg/ml (Fig. 2). Figure 3 shows an effect of pro-CPY addition rate on the refolding efficiency. The refolding efficiency increased with an increase in pro-CPY addition rate, resulting in the highest refolding efficiency of about 18% with a pro-CPY addition rate of 14.4 μg/min. Further increase in the pro-CPY addition rate decreased the refolding efficiency. Hence, it appears that the maximum refolding efficiency can be obtained when pro-CPY addition and pro-CPY cleavage rates are well balanced. The fed-batch refolding process resulted in a refolding efficiency of 18%, which corresponded to a 9-fold increase over that (2%) in the batch process.

In conclusion, a novel fed-batch process was developed to produce an active CPY from pro-CPY expressed as inclusion bodies in *E. coli*. This novel process could be applied for the production of other proteases that are synthesized as zymogens containing a propeptide.

Acknowledgment

This work was supported by a grant from Korea Science and Engineering Foundation through the Center of Advanced Bioseparation Technology (BSEP).

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