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High-Level Secretory Expression of Human Procarboxypeptidase B by Fed-Batch Cultivation of *Pichia pastoris* and its Partial Characterization

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The *procpb* gene encoding human procarboxypeptidase B (proCPB, GeneBank access code AJ224866) was cloned and its *Pichia* expression plasmid, pPIC9α/hproCPB (9.2 kb), was constructed, in which *procpb* was under the control of the *AOX1* promoter and connected to the downstream of the mating factor α-1 (MFα1) signal sequence. The plasmid was linearized by digestion with SacI, and integrated into the genome of *P. pastoris* strain GS115. By culturing of *Pichia* transformant on methanol medium, the human proCPB was successfully expressed and secreted into the culture supernatant. Moreover, Western blot analysis of the extracellular proteins showed proCPB bands clearly at a molecular mass of 45 kDa, confirming the expression of proCPB with its right size. The CPB activity reached about 3.5 U/ml and 12.7 U/ml in the flask and fermentor batch cultures of *Pichia* transformant, respectively. No CPB enzyme activity was found in the intracellular fraction. When the fed-batch cultivation was performed with methanol and glycerol mixture as a feeding medium, the extracellular CPB activity was increased to 42.0 U/ml, which corresponds to a 3.3-fold higher level of CPB activity than that of batch culture. The K_m and k_{cat} values of recombinant human CPB enzyme for hippuryl-L-Arg as a substrate were estimated to be 0.16 mM and 11.93 sec^{-1}, respectively.

Keywords: *Pichia pastoris*, human procarboxypeptidase B, expression, secretion, mating factor α-1

Carboxypeptidases (CPs) remove amino acids from the C-termini of proteins and peptides by hydrolysis. The cleavage mechanisms use an active site serine, cysteine, or zinc; the latter group is referred to as "metallocarboxypeptidases". Altogether, there are 13 known members of the metallocarboxypeptidase gene family in most mammalian species investigated, and an additional carboxypeptidase A-like member (designated CPA3) has been reported in humans [14]. Several additional CP-like genes are present in the human genome, but it is not yet known whether they encode proteins or pseudogenes. All metallocarboxypeptidases can be grouped into one of two major subfamilies based primarily on amino acid sequence similarities. Their participation as proenzymes in the digestive cascade (promoted by limited proteolysis) is a well-documented process. Additionally, their specificity classification between the A forms (CPA, with preference for apolar C-terminal residues) and the B forms (CPB, with preference for basic C-terminal residues) and the tertiary structures of both forms are well known [2, 23].

CPB (E.C. 3.4.17.2) catalyes the hydrolysis of peptides and esters at the C-terminal bond if the terminal residue is either Arg, Lys, or the corresponding α-hydroxyl acids [15]. It also acts at a much slower rate on the C-terminal amino acids Val, Leu, Ile, Asn, Gly, or Gln. CPB is synthesized by the acinar pancreatic cells, which plays an important role in the conversion of proinsulin to insulin [9]. Markvicheva *et al.* [21] applied immobilized trypsin and CPB to prepare human insulin from recombinant proinsulin. Wintersberger *et al.* [28] reported the production of human proinsulin in *Escherichia coli* and the efficient conversion of the renatured proinsulin to mature insulin by treatment with trypsin and CPB. In addition, since proCPB has been known to act as the thrombin-activatable fibrinolysis inhibitor (TAFI), the significance of it as a drug target for thrombolytic therapies is increasing remarkably [1]. Furthermore, if a large quantity of proCPB could be obtained, it is very useful for structure-based drug design.

However, it is worth mentioning that pancreatic CPBs and their precursor are difficult to express in native and

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soluble forms in *E. coli*. Reports about the expression of their precursor forms in eukaryotic cells have indicated a moderate yield (<10 mg/l) until now. The experiments designed to establish an easy and robust expression system capable of providing large amounts of soluble human proCPB were restricted to shake-flask cultures.

The methylotrophic yeast *Pichia pastoris* has been successfully used for the recombinant expression of many foreign proteins [6]. There are many advantages of this system, including the use of the alcohol oxidase I gene (*AOX1*) promoter, the ability to integrate expression plasmids at specific sites of the *Pichia* genome, and the ability to grow cells at high cell-density [6, 8, 13, 16, 19, 20]. Similar to mammalian and insect cells, *P. pastoris* can carry out some co- and post-translational modifications of foreign proteins, and its products are usually obtained with the correct disulfide bonds. Furthermore, in this expression system, the recombinant products can usually be purified to high homogeneity with simple procedures since they are secreted to the extracellular medium, with very low levels of endogenous proteins and enrichment of the overexpressed protein.

In this paper, we describe the construction of expression system and optimization of fermentation processes for the bioactive and extracellular production of human proCPB in *P. pastoris*, and the partial characterization of its enzymatic properties.

MATERIALS AND METHODS

Yeast Strain and Plasmid

For the amplification and subcloning of plasmid DNA and the human proCPB gene (*procpb*), *E. coli* DH5α and pGEM-T-Easy cloning vector (Promega, Madison, WI, U.S.A.) were used. The yeast *P. pastoris* strain GS115 (*his4*, Mut$^+$) and expression vector pPIC9α were purchased from Invitrogen (San Diego, CA, U.S.A.). This yeast expression vector contains the promoter and terminator of the alcohol oxidase I gene (*AOX1*) as an expression cassette and *HIS4* as a selectable marker. The vector pPIC9α has the signal sequence from *Saccharomyces cerevisiae* α-mating factor (MFα1).

Construction of Expression Plasmid

The human proCPB cDNA encoding the proCPB protein fused to a part of the C-terminal of MFα1 was amplified from a human pancreatic cDNA library (Benebiosis Co., Seoul, Korea) by PCR before cloning into the pPIC9α vector. The PCR primers were designed to have proper restriction enzyme sites at the 5' and 3' ends, and synthesized as the following sequences: 5'-CGGAATT-CAAGGTGTTCCGTGTTAACGTTGAAG-3' and 5'-GCTTAGACT-AGTACAGGTGTTCCAGGACGTAGCT-3'. PCR experiments were carried out under conditions with 30 sec at 94°C, 30 sec at 59°C, and 1 min at 72°C for 30 cycles. The PCR-amplified fragment was sequentially purified and cloned into the pGEM-T-Easy cloning vector. After XhoI-EcoRI digestion, the *procpb* gene was ligated in-frame into the XhoI/EcoRI sites of the pPIC9α vector. The constructed plasmid pPIC9α/hproCPB was propagated in *E. coli* DH5α and isolated using the plasmid purification kit (Promega, Madison, WI, U.S.A.). The junction between the MFα1 signal sequence and proCPB was confirmed by the ABI 377 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions.

Transformation of *P. pastoris*

The plasmid pPIC9α/hproCPB was linearized by digestion with SacI to favor integration *via* homologous recombination at the *AOX1* locus of the *Pichia* genome. This DNA segment was used to transform the yeast *P. pastoris* strain GS115 by electroporation at 8 μF, 330 Ω, and 2,000 V with a Bio-Rad GenePulser II (Hercules, CA, U.S.A.). As the corresponding control, the *Pichia* host cell was also transformed with the vector pPIC9α using the same condition. Immediately after pulsing, 1 ml of cold 1 M sorbitol was added to the *P. pastoris* cells. His$^+$ transformants were selected on MD plates (13.4 g of yeast nitrogen base without amino acids (YNB) [Difco Laboratories, Detroit, MI, U.S.A.], 4 μg of biotin, 20 g of dextrose, 15 g of agar, per liter) supplemented with 1 M sorbitol.

Media and Culture Conditions for *P. pastoris* Transformants

P. pastoris transformants were inoculated into 50 ml of BMGY medium (13.4 g of YNB, 4 μg of biotin, 10 g of glycerol per liter of 100 mM potassium phosphate buffer [pH 6.0]) in 500-ml Erlenmeyer baffled-flasks. The precultures were grown on a shaker (150 rpm) at 30°C to an OD_{600} of 5–6. The cells were harvested by centrifugation at 1,500 ×*g* for 10 min and resuspended in 50 ml of BMMY medium (the same as BMGY, except that glycerol was replaced by methanol [5 ml/l] and the medium was supplemented with yeast extract [10 g/l] and peptone [20 g/l]) in 500-ml Erlenmeyer baffled-flasks. The cultures were grown at 30°C under the same aerobic conditions. To enable methanol induction of the *AOX1* promoter, 0.5% methanol was fed every 12 h during the culture period. The growth of transformants was periodically monitored by measuring the OD_{600}. The culture aliquots were collected daily and cells were removed by centrifugation at 3,000 ×*g* for 10 min. The supernatants obtained were used for the assay of CPB activity and then the transformant showing the highest expression level of proCPB was selected.

Fermentation Conditions

The clone exhibiting the highest expression level of hproCPB was used for the batch and fed-batch fermentations, which were carried out in a 3-l fermentor (KoBiotech Co., Incheon, Korea). The seed culture was started from the fresh glycerol stock and directly inoculated into a 500-ml baffled-flask (50 ml working volume) containing BMGY medium. After 24 h of growth, the seed culture of 1% (v/v) was transferred into 50 ml of BMGY medium for the preculture. After 12–16 h of growth, the preculture was used to inoculate into the bioreactor. The fed-batch cultivation was carried out using a mineral medium with the following composition per liter: 0.2 g of $CaSO_4$, 3 g of $MgSO_4 \cdot 7H_2O$, 0.8 g of KOH, 3.5 g of K_2SO_4, 40 g of glycerol, 10 ml of H_3PO_4, and 1 ml of a biotin solution (0.0004%). The biotin was sterilized separately by microfiltration (0.2-μm filter). The fermentation conditions were the following: agitation speed of 800 rpm, temperature of 30°C, pH controlled at 6.0 by adding NH_4OH 30% (v/v), and dissolved oxygen controlled above 30% air saturation at the agitation speed of 400 rpm with an

air flow rate between 1.5 and 2.0 vvm. The glycerol (1%) and methanol (0.5%) mixture was added intermittently in the transition and gene expression phases.

Electrophoresis and Western Blot Analysis

The culture broths collected were centrifuged at 3,000 ×*g* for 10 min, and then the supernatant was used for the measurement of extracellular activity. The intracellular fractions of yeast were obtained by treatment of Zymolyase 100T (Seikagaku Kogyo, Japan) and glass beads. The proCPB expressed in the culture supernatants in the fed-batch culture was analyzed by electrophoresis on 14% polyacrylamide gels in the presence of SDS [17]. The Western blot analysis was performed using rabbit antibody-carboxypeptidase B (Sigma Chemicals Co.) after dilution of 1/1,000 times [24]. The reacting antibodies were detected with anti-rabbit immunoglobulins conjugated to alkaline phosphatase (Sigma Chemicals Co., St. Louis, MO, U.S.A.). Porcine CPB was purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.) and used as a standard for the comparison of molecular weight.

Determination of Enzyme Activity and Kinetic Parameters

The proCPB activities in the extracellular and intracellular fractions were measured by the method of Folk *et al.* [12] with some modifications. The 100 µl of recombinant proCPB in activation buffer (50 mM Tris-HCl, 1 µM $ZnCl_2$, pH 5.0) was treated with 5 U/l trypsin at 37°C for 1 h. The reaction was stopped by adding 1 mM PMSF to the reaction mixture, and aliquots were removed for activity measurements. For activity measurements, 100 µl of the activation mixture was mixed with 900 µl of activation buffer containing 1 mM hippuryl-L-Arg at 25°C and spectrophotometric activity measurements were carried out at 254 nm. One unit of CPB activity was defined as the amount of the enzyme hydrolyzing 1 µmol of hippuryl-L-Arg in 1 min at pH 7.5 and 25°C [11].

The proCPB was partially purified by ultrafiltration by using centricon PL-30 (30,000 MW cut off; Millipore Co., Billerica, MA, U.S.A.). The kinetic parameters, V_{max}, K_m, and k_{cat}, of the enzyme were calculated over the concentration ranges of 0.1–1.0 mM substrates by a Lineweaver-Burk plot. The measurement of initial reaction rates was carried out with the substrates hippuryl-L-Arg, hippuryl-L-Lys, and *N*-(3-[2-furyl]acryloyl)-Ala-Lys (Sigma Chemicals Co.).

Results and Discussion

Construction of Human proCPB Expression Plasmid in *P. pastoris*

The human pancreatic cDNA library was used for PCR amplification of the proCPB-encoding gene without the native signal sequence (prepeptide region), and yielded a 1.2 kb DNA fragment containing a partial segment of the MFα1 signal sequence and the whole coding region of the proCPB sequence. After digestion with XhoI-EcoRI restriction enzymes, the *procpb* gene was ligated in-frame into the XhoI/EcoRI sites of the pPIC9α vector. The constructed plasmid pPIC9α/hproCPB (9.2 kb, Fig. 1A) was linearized by digestion with SacI and was transformed into the *P. pastoris* strain GS115. The integration of the proCPB coding region into the *P. pastoris* genome was confirmed by PCR with the colonies selected on MD plates.

By DNA sequencing of pPIC9α/hproCPB, the junction between the MFα1 signal sequence and proCPB was confirmed as expected (Fig. 1B). The sequence of original target for the removal of the MFα1 propeptide is KREAEAEA, which is cleaved after the dibasic peptide KR by the yeast endopeptidase KEX2 and undergoes subsequent elimination of Glu-Ala dipeptides by dipeptidyl aminopeptidase A [18]. Thus, according to the design of the pPIC9α/hproCPB expression plasmid, the prosegment of MFα1 is expected to be cleaved after the dibasic residues Lys-Arg by a single KEX2 endopeptidase in *P. pastoris* [22], and the proCPB coding region can keep its own amino acid sequence starting with HHGGE (Fig. 1B).

Selection of *Pichia* Transformant and Expression of proCPB in the Shake-Flask Culture

After electroporetic transformation of the host *P. pastoris* strain GS115 with pPIC9α/hproCPB, 10 clones were randomly selected on MD medium plate. Through 10-ml

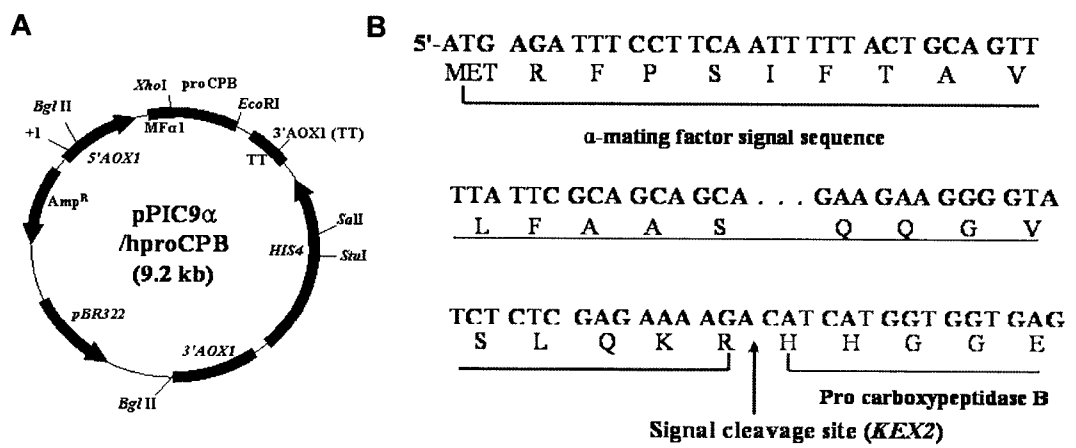

Fig. 1. *A*.Schematic diagram of human proCPB expression plasmid, pPIC9α/hproCPB. *B*. Nucleotide sequence and deduced amino acid sequence of the junction site between the MFα1 signal sequence and proCPB.

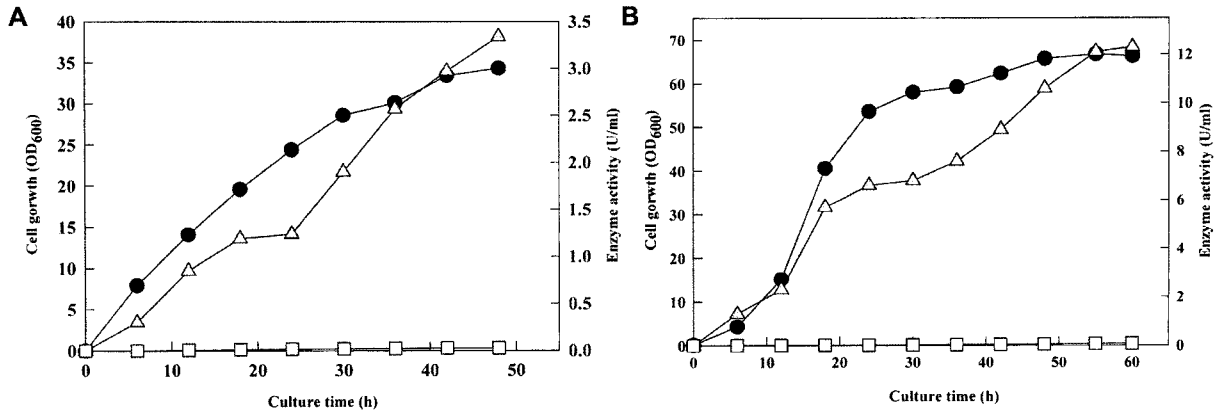

Fig. 2. Time profiles of cell growth and proCPB expression in the baffled-flask culture (**A**) and in the fermentor batch culture (**B**) of *P. pastoris* pPIC9α/hproCPB.
Symbols: (●), cell growth; (▲), carboxypeptidase B activity in the culture supernatant; (□), intracellular carboxypeptidase B activity.

test tube culture, these clones were tested for their ability to secrete proCPB following methanol induction by performing a CPB activity assay. The selected clone with the highest expression level was chosen for the subsequent shake-flask culture experiment. Fig. 2A shows the cell growth and hproCPB activity in the baffled shake-flask. The cell growth and total activity of proCPB continuously increased and reached about 34 OD_{600} and 3.52 U/ml, respectively, until 48 h of cultivation on BMMY medium, and most of the proCPB activity (95%) was found in the extracellular medium (3.34 U/ml).

Expression of proCPB in the Fermentor Batch Culture

A *Pichia* fermentation process with feeding of 0.5% methanol every 12 h during the culture period was conducted. Fig. 2B shows the time profiles of cell growth (OD_{600}), and intracellular and extracellular activities of proCPB during the fermentor batch culture. The proCPB activity increased after methanol induction at 12 h and was raised gradually through 60 h of cultivation.

The cell growth reached a maximum 65 OD_{600}, and the total proCPB activity was 12.7 U/ml, in which the extracellular and intracellular activities were 12.3 U/ml and 0.45 U/ml, respectively, at 60 h. Compared with the shake-flask culture, a significantly increased expression level of recombinant proCPB enzyme might be resulted from the higher cell density in the fermentor batch culture of *P. pastoris*. About 3.6-fold increase in the extracellular expression level of proCPB was obtained when the culture scale was changed from the shake-flask to the bioreactor, which provides

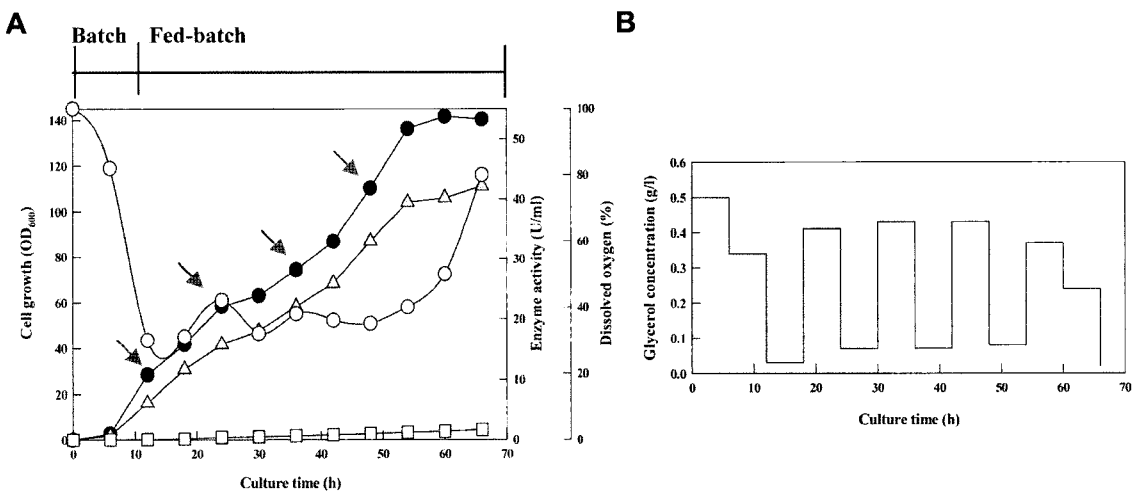

Fig. 3. Time profiles of cell growth, proCPB expression (**A**), and glycerol concentration (**B**) in the fed-batch fermentation of *P. pastoris* pPIC9α/hproCPB.
Symbols: (●), cell growth; (▲), carboxypeptidase B activity in the culture supernatant; (□), intracellular carboxypeptidase B activity; (○), dissolved oxygen (%); (↘), feeding of 1% methanol and 0.5% glycerol.

the controlled conditions of pH 6.0 and DO level above 30% air saturation, and leads to a higher cell growth.

High-Level Expression of proCPB in the Fed-Batch Fermentation

Fig. 3 shows the result of fed-batch cultivation employing BMY medium and additional feeding of glycerol and methanol. After a batch growth phase using glycerol as the single carbon source, in which the depletion of glycerol was indicated by a sharp increase of the dissolved oxygen level, a transition phase with simultaneous feeding of glycerol and methanol at the time of DO increase was started. The feeding strategy was accorded to a modified scheme of Carmen *et al.* [5] and Katakura *et al.* [16]. During the transition phase and gene expression phase, glycerol was always maintained at the limiting concentrations (*i.e.*, below the detection limits). The batch and fed-batch processes were continued for 66 h. The enzyme activity of intracellular and extracellular proCPB increased with increasing the cell density. The secreted proCPB activity reached about 42.0 U/ml at 66 h of cultivation. The maximum secretion level of the enzyme activity in the fed-batch culture was enhanced 3.3-fold over that of batch cultivation.

Western blot analysis of the extracellular fractions of the fed-batch culture showed that the hproCPB expression strain, GS115/pPIC9α-hproCPB, secreted a large amount of recombinant proCPB protein that was visible after 12 h of cultivation (Fig. 4). The molecular mass (M_r) of the protein was estimated to be approximately 45 kDa. No band corresponding to about 45 kDa was observed in the Western blot analysis of the culture supernatant of *P. pastoris* host GS115. Following treatment with trypsin, the M_r of this proCPB band decreased to about 34 kDa, which is consistent with the calculated M_r of mature CPB. The mass of CPB, 34 kDa, also revealed that an increase of M_r by post-translational modification such as glycosylation did not occur in the human CPB polypeptide. In fact, human CPB has no N-linked glycosylation site (Asn+X+Thr/Ser, X=any amino acid). This maturation or M_r difference after trypsin treatment

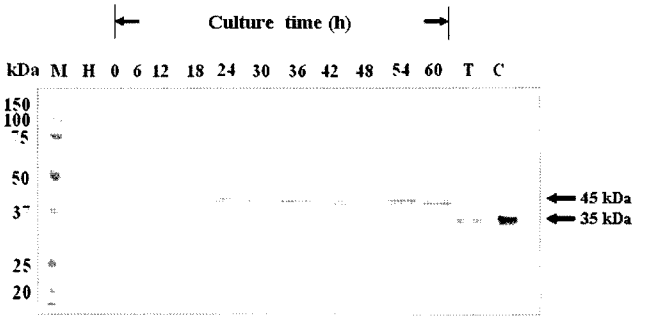

Fig. 4. Western blot analysis of proCPB expressed in the fed-batch culture of *P. pastoris* pPIC9α/hproCPB.
Lane M; protein molecular mass marker; lane 2, *P. pastoris* host GS115; lane 3–12, extracellular samples after 0–60 h culture in BMMY; T, removal of pro-region from proCPB by trypsin treatment; C, porcine CPB.

was also observed in the porcine proCPB [26]. These results strongly suggested that hproCPB in *P. pastoris* was successfully expressed and secreted as a pro form of CPB.

As reported in this paper, the recombinant fermentation process could be successfully regulated by feeding of glycerol and methanol, so that a large amount of proCPB enzyme could be produced as a secreted form in the *Pichia* cell. A 10-fold enhanced production of heterologous proteins in *P. pastoris* could be achieved by high cell-density fermentation. Through the high cell-density fed-batch cultivation of *Pichia* transformants, porcine proCPB was expressed at a high yield (250 mg/l) [26] and the production level of rat proCPB reached 500 mg/l in the culture supernatant [27]. Moreover, the acetyl xylan esterase gene from *Aspergillus ficuum* was overexpressed at the level of 930 U/ml (9.8 g/l) [20], and the lipase from *Yarrowia lipolytica* could be expressed at the high level of 12,500,000 U/l (0.63 g/l) [7].

Partial Characterization of Recombinant proCPB

With the partially purified proCPB from the extracellular fraction, kinetic studies were performed and the V_{max} and K_m values for the different substrates were estimated by Lineweaver-Burk plotting (Fig. 5). Kinetic parameters for

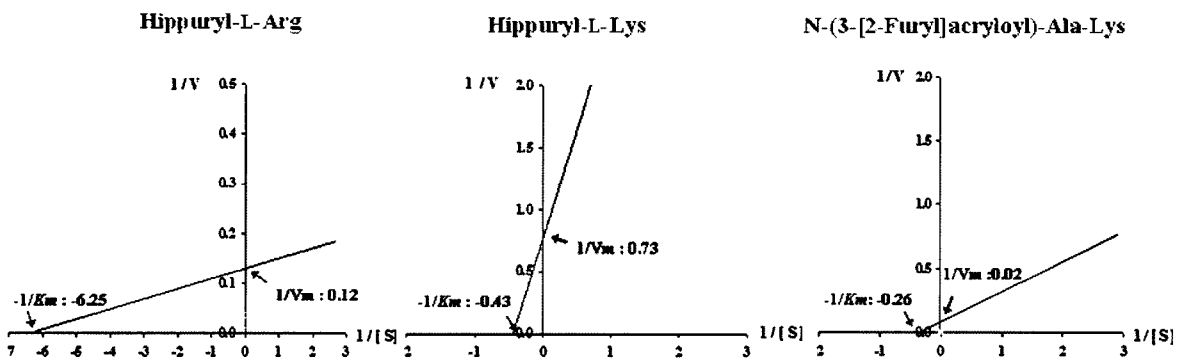

Fig. 5. Lineweaver-Burk plots of human proCPB expressed in *Pichia*, in which hippuryl-L-Arg, hippuryl-L-Lys, and *N*-(3-[2-furyl]acryloyl)-Ala-Lys were used as substrates.

Table 1. Comparison of V_{max}, K_m and k_{cat} values of recombinant human proCPB when hippuryl-L-Arg, hippuryl-L-Lys, or *N*-(3-[2-furyl]acryloyl)-Ala-Lys were used as a substrate.

Substrate	V_{max} (μmol/ml·min)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($sec^{-1}·mM^{-1}$)
Hippuryl-L-Arg	8.13	0.16	11.93	74.56
Hippuryl-L-Lys	1.37	2.31	90.7	39.26
N-(3-[2-Furyl]acryloyl)-Ala-Lys	50.00	3.83	33.3	8.69

the hydrolysis of hippuryl-L-Arg, hippuryl-L-Lys, and *N*-(3-[2-furyl]acryloyl)-Ala-Lys were determined at 25°C, pH 8.0 (Fig. 5). The results indicate that the CPB enzyme is more efficient with hippuryl-L-Arg (0.16 mM) than with hippuryl-L-Lys (2.31 mM) and *N*-(3-[2-furyl]acryloyl)-Ala-Lys (3.83 mM). This K_m value for hippuryl-L-Arg as substrate was similar to that (0.18 mM) of the human CPB expressed in *E. coli* [10]. In the case of human CPB, the K_m value for the hippuryl-L-Arg substrate was about 2- to 3.4-fold lower than the reports of other species (porcine CPB=0.39 mM and ostrich CPB=0.54 mM) [3]. Moreover, from the k_{cat}/K_m ratios for the different substrates, we can conclude that hippuryl-L-Arg is the most efficient substrate for the recombinant human CPB.

Consequently, the *Pichia* system developed in this study allowed us to produce extracellularly large amounts of the human proCPB enzyme, which amouts might be sufficient for the conversion of proinsulin to insulin and for the development of specific antibodies and antagonists. In addition, the partial characteristics or kinetic parameters of recombinant proCPB would be useful for the structure-based design of drugs targeting thrombolytic therapies.

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