High expression of human carboxypeptidase M in *Pichia pastoris*. Purification and partial characterization

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Abstract

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Carboxypeptidase M (CPM) is an extracellular glycosylphosphatidyl-inositol-anchored membrane glycoprotein, which removes the C-terminal basic residues, lysine and arginine, from peptides and proteins at neutral pH. CPM plays an important role in the control of peptide hormones and growth factor activity on the cell surface. The present study was carried out to clone and express human CPM in the yeast *Pichia pastoris* in order to evaluate the importance of this enzyme in physiological and pathological processes. The cDNA for the enzyme was amplified from total placental RNA by RT-PCR and cloned in the vector pPIC9, which uses the methanol oxidase promoter and drives the expression of high levels of heterologous proteins in *P. pastoris*. The cpm gene, after cloning and transfection, was integrated into the yeast genome, which produced the active protein. The recombinant protein was secreted into the medium and the enzymatic activity was measured using the fluorescent substrate dansyl-Ala-Arg. The enzyme was purified by a two-step protocol including gel filtration and ion-exchange chromatography, resulting in a 1753-fold purified active protein (16474 RFU mg protein^{-1} min^{-1}). This purification protocol permitted us to obtain 410 mg of the purified protein per liter of fermentation medium. SDS-PAGE showed that recombinant CPM migrated as a single band with a molecular mass similar to that of native placental enzyme (62 kDa), suggesting that the expression of a glycosylated protein had occurred. These results demonstrate for the first time the establishment of a method using *P. pastoris* to express human CPM necessary to the development of specific antibodies and antagonists, and the analysis of the involvement of this peptidase in different physiological and pathological processes

Introduction

Carboxypeptidase M (CPM) is an extracellular glycosylphosphatidyl-inositol-anchored membrane glycoprotein. This protein is a member of the CPN/E subfamily of zinc metallo-carboxypeptidase. It specifically removes C-terminal basic residues such as lysine and arginine from peptides and from proteins at neutral pH (1-4). The highest levels of CPM have been found in human lung and placenta, but significant amounts are present in kidney, blood vessels, intestine, brain, and peripheral nerves (1,5-7). CPM has been found also in soluble form in various body fluids, including amniotic fluid, seminal plasma and urine (5,8,9). Due to its wide distribution in a variety of tissues, it is believed to play important roles in the control of peptide hormone and growth factor activity on the cell surface, and in the membrane-localized degradation of extracellular proteins (10).

By removing the C-terminal Arg residue of the intact kinins (and related peptides), CPM can switch the receptor specificity (11), inactivating the agonist for the B2 receptor and concomitantly producing an agonist for the B1 receptor (1). Recent results from our group have shown that the kinin B1 receptor is important for neutrophil migration into inflamed tissues (12,13) and for glucose/insulin homeostasis (Araujo RC, unpublished data). Furthermore, CPM is recognized by antibodies that detect cell surface antigens on macrophages and is strongly induced in the differentiation of monocytes to macrophages (14). Therefore, the mechanism for neutrophil migration could be dependent on the CPM present in the cell membrane of macrophages, indicating a relevant role of this enzyme at sites of inflammation. In addition, the conversion of agonistic activity was shown to result in an enhanced and prolonged output of nitric oxide in response to bradykinin or kallidin in cytokine-stimulated human lung microvascular endothelial cells (15). By cloning the cDNA from different human tissues, we could determine the molecular structure (16) and show the presence of alternatively spliced messages for CPM. Furthermore, we could also show that the expression of this carboxypeptidase in tumor tissues is differently regulated, raising the question of the role of this enzyme in cancer (16). Thus, taking into account the function played by kinins and nitric oxide in inflammatory diseases and cancer (11), and the fact that specific inhibitors of this carboxypeptidase are still lacking, CPM might be an important target for the development of new drugs to treat these pathologies.

Recently, a human CPM (hCPM) was expressed by using a system based on baculovirus-infected insect cells (Sf9) (17). The cited study aimed to identify the membrane-anchor signal and the glycosylphosphatidyl-inositol attachment site and to investigate the roles of the two conserved glutamic acid residues, Glu^{260} and Glu^{264}, in kinetic parameters and in protein stability. Furthermore, the same group determined the crystal structure of the human enzyme (18).

Therefore, the objective of the present study was to obtain an active glycosylated protein using the expression system of the yeast *Pichia pastoris*. This system has several advantages, including the use of the alcohol oxidase I gene promoter, the ability to stably integrate expression plasmids at specific sites, the ability of the cells to be cultivated at high density, and a simplified purification procedure for secreted heterologous proteins. Moreover, similar to mammalian and insect cells, *P. pastoris* can carry out certain co- and post-translational modifications of foreign proteins (19,20). The results described in the present report demonstrate a high level of expression of the active recombinant hCPM with a molecular mass similar to the native glycoprotein (62 kDa), indicating that the *P. pastoris* expression system can be useful for the production of active CPM at relevant levels.

Material and Methods

Enzymes, primers and vectors for molecular biology procedures were purchased from Invitrogen (Carlsbad, CA, USA) and Promega (Madison, WI, USA). The dansyl-Ala-Arg substrate was from Bachem (King of Prussia, PA, USA). DNA sequencing was performed using an ABI377 sequencer (Applied Biosystems, Foster City, CA, USA). The Amicon Ultra 30,000 MWCO membrane was from Millipore (Billerica, MA, USA), and the Superdex 200 HR 10/30 and Mono Q HR 5/5 columns were from Amersham Biosciences (Piscataway, NJ, USA).

RNA extraction and reverse transcription polymerase chain reaction

Total RNA from human placenta was isolated using the Trizol reagent according to the manufacturer's protocol and its purity was evaluated by electrophoresis on 1% agarose gel. Contamination of RNA samples with genomic DNA was avoided by treatment for 1 h at 37ºC with 1 U RNAse-free/DNAse I per 2 µg RNA. For the same amount of RNA, the reaction also contained 20 U RnaseOUT-Rnase inhibitor and 3 mM $MgCl_2$. After incubation, samples were heated to 95ºC and immediately chilled on ice for DNAse I denaturation. Reverse transcription was performed using 2 µg total RNA, 200 U Moloney murine leukemia virus II reverse transcriptase, 5 mM DTT, 50 ng random hexamer primers, 1X PCR buffer, 0.5 mM dNTPs, and 3 mM $MgCl_2$. Reactions were carried out at 20ºC for 10 min, 42ºC for 45 min, 95ºC for 5 min, and 4ºC for 10 min. The resulting cDNA from the human placenta was then used for PCR which was performed with 500 ng cDNA. The PCR mixture contained 0.5 µM concentrations of sense (hCPM5 5'-GAATTCTTGCTGCCTT TGGTAGCTGC) and antisense (CPMAS2 5'-GCGGCCGCTTATTTGAAGAATAT GTGC) primers, which contained added sites for the restriction enzymes *Eco*RI and *Not*I, respectively, 2 U platinum TaqDNA polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl, pH 8.3, in a 50-µL volume. PCR amplification was performed by incubating the samples at 94ºC for 1 min, followed by 30 cycles at 94ºC for 1 min, and at 60ºC for 90 s, with a final extension at 72ºC for 7 min. At the end of amplification, samples were submitted to electrophoresis on 1% agarose gel with a 100-bp DNA ladder as a size marker. The amplified bands of 1313 bp were visualized by ethidium bromide staining.

Construction of expression vector pPIC9-cpm

The PCR-amplified fragment encoding hCPM was cloned into the pGEM-T-Easy (Promega) cloning vector and subjected to double-stranded DNA sequencing (ABI 377, Applied Biosystems) After *Eco*RI-*Not*I digestion, the cpm gene was cloned in frame into the pPIC9 (Invitrogen) vector between the *Eco*RI (5' end) and *Not*I (3' end) restriction sites to generate the plasmid pPIC9-cpm.

Transformation of *Pichia pastoris* and selection of high-level expression colonies

The following culture media used for transformation of *P. pastoris*, selection of recombinant clones, and expression of CPM were prepared according to manufacturer recommendations (Invitrogen): minimal dextrose medium (MD), minimal methanol medium (MM), buffered glycerol-complex medium (BMGY), and buffered minimal methanol (BMMY). For cultures in liquid BMMY, which contain methanol as an inducer and carbon source, methanol was added every 24 h to a final concentration of 0.5% (v/v). All cultures were carried out at 30ºC. The pPIC9-cpm construct (10 µg) was linearized with *Bgl*II and transformed into *P. pastoris* wild-type strain GS115 (Invitrogen)

by electroporation at 25 µF, 400 Ω, and 1500 V using a Bio-Rad (Hercules, CA, USA) GenePulser. Immediately after pulsing, 1 mL cold 1 M sorbitol was added to the *P. pastoris* cells, and plated onto MD containing 1 M sorbitol. After cultivation, all transformants were patched or replica plated onto both MM and MD plates to select the colonies growing more slowly in MM than in MD, and these colonies were cultivated in liquid BMMY. To analyze cpm gene integration into the *P. pastoris* genome, genomic DNA was isolated from different clones and integration was confirmed by PCR using the same cloning primers. CPM activity of positive clones in the BMMY supernatant was measured with the dansyl-Ala-Arg substrate as previously described (21). The clone presenting the highest CPM activity was inoculated into 100 mL BMGY and cultured for 1 day. The cells were then collected by centrifugation and resuspended in 500 mL BMMY. Every day (0, 24, 48, 72, and 96 h), just before the addition of 0.5% methanol, an aliquot of the medium was collected to measure CPM activity and protein concentration.

Purification of recombinant carboxypeptidase M

The CPM secreted in the supernatant was purified by a two-step procedure consisting of gel filtration and ion-exchange chromatography. Briefly, the expression medium was concentrated on an Amicon concentrator with an Amicon Ultra 30,000 MWCO membrane (Millipore), and loaded on a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with 50 mM Na-phosphate buffer, pH 7, containing 0.15 M NaCl. The column was eluted with the equilibration buffer at a flow rate 0.8 mL/min, and 0.5-mL fractions were collected and assayed for CPM activity. The effluent from the gel filtration column containing enzyme activity was combined and loaded

onto a Mono Q HR 5/5 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl buffer, pH 8. The column was washed with equilibration buffer and then eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. Proteins were detected by UV absorption at 280 nm, 0.5-mL fractions were collected and assayed for CPM activity.

Protein concentration was determined by the dye-binding method of Bradford (22) using bovine serum albumin as a standard.

Results and Discussion

Analysis of transformed clones and expression of human carboxypeptidase M

Total RNA from human placenta was isolated and submitted to the reverse transcription. The resultant cDNA was then used for PCR and yielded a 1313-bp DNA fragment containing the whole coding region with the expected sequence, except for one base change at position 1158 (T → A; data not shown). However, this base alteration does not lead to a change in the amino acid sequence of the generated protein. After digestion with *Eco*RI-*Not*I restriction enzymes, the cpm gene was cloned in frame in the pPIC9. The generated plasmid pPIC9-cpm containing the hCPM gene was transformed into the *P. pastoris* wild-type strain GS115. Some of the colonies selected on MD plates (10 of 34 colonies) were tested by PCR to confirm the integration of the CPM coding region into the *P. pastoris* genome and six positive clones were selected. The clone with the highest activity (9.4 RFU min^{-1} mg protein^{-1}) and one negative control, pPIC9 (without the insert), were initially inoculated into BMGY and later into BMMY. The cells and the supernatant of these clones were then collected by centrifugation, CPM activity was measured and protein concentration was determined. As shown in Figure 1, carboxypeptidase activity was detected at high levels in the supernatant of the positive clone.

Figure 2 shows the levels of the total carboxypeptidase activity at different times. As observed, CPM activity increased in the medium in a time-dependent manner. The advantage of expressing heterologous proteins as secreted proteins is that *P. pastoris* secretes very low levels of native proteins. This, combined with the very low amount of protein in the minimal *Pichia* growth medium, means that the medium serves as the first step in the purification of the protein (19).

Purification of human carboxypeptidase M

The supernatant was concentrated through an Amicon membrane and submitted to gel filtration chromatography (Superdex 200 HR 10/30). Protein elution was monitored by measuring the absorbance at 280 nm and the carboxypeptidase activity was evaluated with the substrate dansyl-Ala-Arg. Protein eluted from the column showed one major peak of activity with a molecular mass estimated between 40 and 90 kDa (Figure 3A). The fractions corresponding to this major peak were pooled (P) and submitted to SDS-PAGE. The results showed the presence of three main bands in the gel (Figure 3B). The active fraction was submitted to ion-exchange chromatography (Mono Q 5/5) which resulted in only one peak of activity and a band in SDS-PAGE around 62-70 kDa (Figure 4), similar to the 62 kDa corresponding to the native placental protein. This small difference in molecular mass could be attributed to the partial cleavage produced by the *P. pastoris* STE13 protease in the recombinant protein, leaving four additional amino acids (EAEA) at the N-terminal region (23). Another reason could be the differences in the number and type of sugar units added to the yeast-secreted proteins compared with human proteins (24).

The purification steps resulted in 1753-fold purification of recombinant hCPM. The specific activity of the purified enzyme was
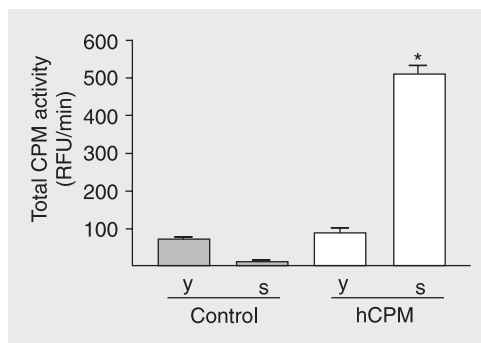

Figure 1. Expression of recombinant hCPM in the control and transformed (hCPM) clones of *Pichia pastoris* 24 h after inoculation into buffered glycerol-complex medium. The yeast cells (y) and the supernatant (s) were separated by centrifugation and assayed for CPM activity with dansil-Ala-Arg as substrate. hCPM = human carboxypeptidase M; RFU = relative fluorescence units. *$P < 0.01$ compared to the supernatant of the control clone (*t*-test).
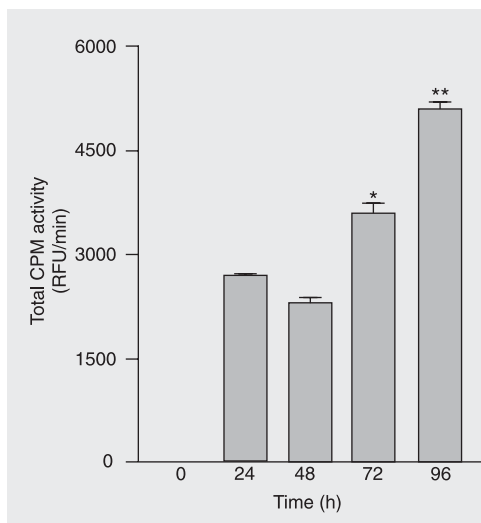

Figure 2. Carboxypeptidase M activity in the culture supernatant of the GS115-CPM strain. Cells were induced in buffered minimal methanol and the activity was evaluated with the substrate dansil-Ala-Arg at different times. RFU = relative fluorescence units. *$P < 0.05$ and **$P < 0.01$ compared to the 24-h culture (*t*-test).
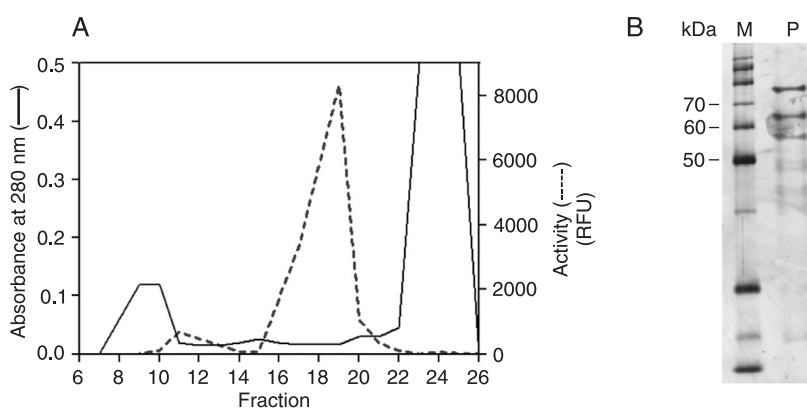

Figure 3. *A*, Gel filtration of recombinant human carboxypeptidase M (CPM). The concentrated supernatant (3.05 mg/mL in 2 mL) was loaded onto a Superdex 200 HR 10/30 column in the FPLC system. The column was equilibrated with 50 mM Na-phosphate buffer, pH 7, containing 0.15 M NaCl and eluted with the equilibration buffer at a flow rate of 0.8 mL/min. Fractions of 0.5 mL were collected and assayed for CPM activity. RFU = relative fluorescence units. *B*, SDS-PAGE (12%) of pooled fractions containing CPM activity (P). M, molecular mass markers (Bench Mark Protein Ladder, Invitrogen).

16474 RFU mg^{-1} min^{-1} using dansyl-Ala-Arg as substrate (Table 1). The very low levels of specific activity observed for the crude supernatant when compared to the purified enzyme may be explained by the presence of inhibitors of the enzyme in the fermentation medium. Some salts added to the medium at high concentrations are able to inhibit CPM and probably are separated from the enzyme during the purification process, causing a large increase in specific and total activities.

The latest achievements in the expression system of *P. pastoris* have changed deeply the methodology used to express heterologous proteins, increasing the expression levels and bioactivity of the latter. In addition, the great success of *P. pastoris* is related to the fact that this organism is a eukaryote, capable to express proteins with post-translational modifications, sometimes essential for proper function. A further benefit of the *P. pastoris* system is that strong promoters are available to drive the expression of the foreign gene(s), thus enabling production of large amounts of the target protein(s) with relative technical ease and at a lower cost than most other eukaryotic systems (25).

The results shown here demonstrate the establishment of a method using *P. pastoris* to express CPM in high yield of purified enzyme, i.e., 410 mg/L of fermentation culture. The expression levels of the hCPM in *Pichia* obtained in the present study was much higher (around 10-fold) when compared to the published expression system using baculovirus-infected insect cells, about 30-40 mg/L of recombinant mutant CPM in the culture medium (17). Some investigators, using the same system, have also described a similar recovery for the enzyme tannin acyl hydrolase (25), and even higher recoveries for the enzyme lipase/acyltransferase from *Candida parapsilosis* (EC 3.1.1.3) (26), and for the tetanus toxin fragment C (24,27), highlighting the potential of this technique to express high yield of heterologous proteins.

The present data show that we developed a methodology that permits the production of large amounts of the CPM enzyme, necessary for the development of specific antibodies and antagonists and for the analysis of the involvement of this peptidase in different physiological and pathological processes.
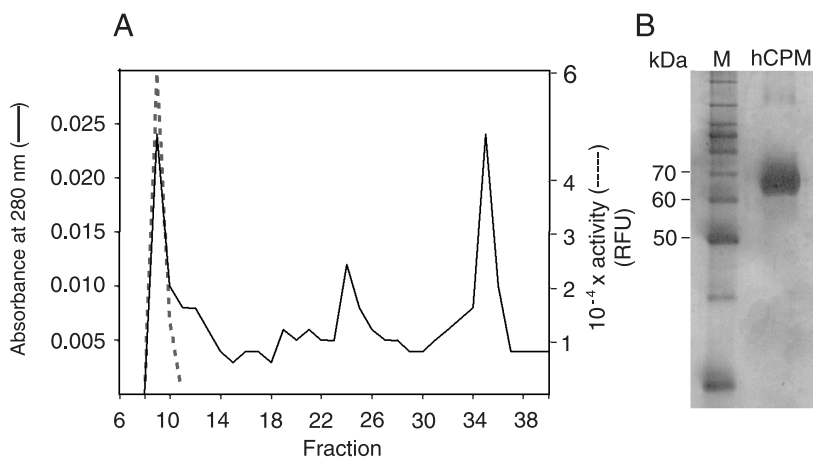

Figure 4. *A*, Ion-exchange chromatography. A pool of effluent containing enzyme activity (0.28 mg/mL in 1.5 mL) obtained by gel filtration (Figure 3) was loaded onto a Mono Q HR 5/5 column equilibrated with 50 mM Tris-HCl buffer, pH 8. The column was washed with equilibration buffer and then eluted with a linear gradient (0 to 1 M) NaCl in the same buffer. Proteins were detected by UV absorption at 280 nm. Fractions of 0.5 mL were collected and assayed for carboxypeptidase M activity. RFU = relative fluorescence units. *B*, SDS-PAGE (12%) of pooled active fractions. M, molecular mass markers (Bench Mark Protein Ladder, Invitrogen). Human carboxypeptidase M (hCPM) appears as a diffuse band because it is a glycoprotein.

Table 1. Purification of recombinant hCPM from 1 mL of fermentation supernatant.

Step	Protein (mg)	Total activity*	Specific activity**	Purification (X-fold)
Supernatant	6.10	57.3	9.4	1
Superdex 200 HR	0.86	2077.0	2415	257
Mono Q	0.41	6754.3	16474	1753

*RFU/min. **RFU min^{-1} mg protein^{-1}. RFU = relative fluorescence units; hCPM = human carboxypeptidase M. Purification = Mono Q specific activity/supernatant specific activity.

Acknowledgments

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