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Cloning, expression, and activity analysis of human cathepsin C in the yeast *Pichia pastoris*

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Abstract: The yeast *Pichia pastoris* expression system was investigated for the production of human cathepsin C (CatC) recombinant protein. The full-length CatC cDNA, corresponding to amino acids 12–475, was synthesized from interleukin-2 (IL-2) stimulated human peripheral blood mononuclear cells and subcloned in the pGEM-T cloning vector. After confirming the DNA sequence of the insert, the gene was cloned into the pPICZαA expression vector under the control of the methanol-inducible alcohol oxidase (*AOX1*) promoter and transformed to *P. pastoris* X-33 cells. The expressed protein was secreted into the culture medium through the α-factor mating signal sequence of the expression vector. Analysis of the culture supernatant revealed that the recombinant human CatC was secreted as a 58-kDa molecule, indicating that human CatC was accumulated in the culture supernatant as proform composed of the residual propart, the activation peptide, and the heavy and light chains. Extracellular recombinant proCatC was further activated by cysteine endoprotease papain in vitro and its activity was confirmed by assays using a synthetic substrate.

Key words: Human cathepsin C, *Pichia pastoris*, recombinant protein, expression

1. Introduction

Cathepsin C (dipeptidyl peptidase I, EC 3.4.14.1) is a lysosomal cysteine protease belonging to the papain superfamily (Izumya and Fruton, 1956). Besides its role in lysosomal protein degradation (Coffey and de Duve, 1968), CatC is a key enzyme in the activation of many serine proteinases that are capable of degrading various extracellular matrix components and can lead to tissue damage and chronic inflammation such as chronic obstructive pulmonary disease and asthma (Wolters et al., 2000; Adkinson et al., 2002; Kim and Nadel, 2004). At the same time, related studies have demonstrated that inhibitors of CatC could potentially be useful therapeutics for the treatment of such diseases (Methot et al., 2007, 2008). Therefore, production of recombinant human CatC protein in an effort to develop inhibitors of this enzyme is highly appreciated. In addition to this importance, due to its unique proteolytic specificity in sequential removal of dipeptides from the amino terminus of protein substrates, attention has also been drawn to the industrial applications of CatC as a processing exopeptidase to use in amino acid sequencing of proteins and removal of fusion peptides from the N-termini of recombinant proteins (Lauritzen et al., 1998; Pedersen et al., 1999).

The methylotrophic yeast *Pichia pastoris* is a very successful microbial heterologous protein expression host that can be grown to high cell densities in simple and cheap medium and is capable of secreting large amounts of correctly folded eukaryotic recombinant proteins in simple media (Cereghino and Cregg, 2000). Contrary to bacterial expression systems, *P. pastoris* allows posttranslational modifications such as disulfide bond formation, glycosylation similar to eukaryotic cells, and proteolytic maturation (Cregg et al., 1993). One of the most important benefits of this strain as an expression host is that it does not have papain-like cysteine proteases in its genome. The absence of protease homologs in the host genome majorly simplifies the purification of the recombinant protein (Linnevers et al., 1997).

To date, a number of studies have been reported on recombinant expression of human CatC that were performed in insect and mammalian cell systems (Dahl et al., 2001; Yang et al., 2011). However, these systems are high-cost methods that require complex and expensive media and involve cumbersome work procedures. In this study, the expression pattern of the human CatC gene in the yeast *P. pastoris* was studied for simple and effective protein production. Its cDNA synthesis, recombinant expression, extracellular secretion, proteolytic maturation, and enzymatic activity are reported. It was found that the yeast *P. pastoris* expression system is capable of expressing the proform of the enzyme efficiently, which can be further

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maturated by cysteine endoprotease papain in vitro. To my knowledge, this is the first study reporting human CatC recombinant expression in *P. pastoris*.

2. Material and methods

2.1. Materials

The *Pichia pastoris* expression kit and TRIzol reagent were purchased from Invitrogen. Human peripheral blood mononuclear cells (PBMCs) were provided by Lonza. *E. coli* DH5α was used for construction and propagation of all vectors. The pGEM-T Easy vector was from Promega. DNA restriction enzymes and RT-PCR Kit were from New England Biolabs. Interleukin-2 (IL-2) was from Santa Cruz Biotechnology. Papain from papaya latex and Gly-Phe-*p*-nitroanilide were purchased from Sigma. RPMI-1640 growth medium, 10% fetal bovine serum, streptomycin, and penicillin were from GIBCO Life Technologies. All other chemicals and reagents were of the highest purity.

2.2. CatC cDNA synthesis and pGEM-T-CatC construction

PBMCs were cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 pg/mL streptomycin and stimulated with IL-2 (1000 U / 2 × 10^6 cells/mL) to increase CatC mRNA levels for 48 h at 37 °C under 5% CO_2 (Rao et al., 1997). After incubation, total RNA of the stimulated cells was isolated with TRIzol reagent. Briefly, the stimulated cells were harvested and washed twice with phosphate-buffered saline. The cells were lysed with 1 mL of TRIzol by pipetting and incubated at room temperature for 5 min to dissociate nucleoprotein complexes. Chloroform (0.2 mL) was then added to the lysate and mixed by stirring for 2 min. After centrifugation (at 4 °C and 12,000 × *g* for 15 min), the mixture separated into three phases, consisting of a lower organic phase, an interphase, and an upper aqueous phase. The aqueous phase was transferred to a fresh centrifuge tube in the presence of 0.5 mL of isopropyl alcohol and incubated at room temperature for 10 min. Finally, total RNA was precipitated via centrifugation (at 4 °C and 12,000 × *g* for 10 min), washed twice with 1 mL of 75% ethanol, and dissolved in 50 µL of RNase-free water.

The reverse transcription of total RNA into cDNA was performed using the first-strand cDNA synthesis kit according to the manufacturer's instructions. The CatC-encoding region was amplified from the cDNA pool by PCR using the following primers: forward primer 5'-GGG CGG CCG CAT GGG TGC TGG GCC CTC CT and reverse primer 5'-GGG CGG CCG CCC CTA CAA TTT AGG AAT TGG TGT (*Not*I recognition sequence is underlined). Primers contained *Not*I sites for cloning into the *P. pastoris* expression vector pPICZαA. The PCR amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 40 s, and extension at 72 °C for 100 s; a final extension step of 10 min at 72 °C was added at the end of this reaction. The PCR product was then analyzed on an agarose gel and directly subcloned into the pGEM-T Easy cloning vector using the TA cloning system. The nucleotide sequence of the insert in the resulting pGEM-T-CatC vector was confirmed by DNA sequencing using the T7 and SP6 promoter sequencing primers.

2.3. Construction and transformation of pPICZαA-CatC expression vector

The pGEM-T-CatC cloning vector was transformed into *E. coli* DH5α competent cells using the classic heat shock method and grown overnight at 37 °C on LB plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) containing 50 µg/mL ampicillin. After 24 h, white colonies were selected and the presence of the insert was determined by employing colony PCR. The transformed colonies were then grown overnight at 37 °C and 200 rpm in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 50 µg/mL ampicillin and plasmid purification was performed. In the resultant plasmid, pGEM-T-CatC, the 1.4-kb *Not*I fragment encoding CatC was excised from the vector, ligated into the *Not*I site at the multicloning site of the pPICZαA expression vector, and transformed into DH5α cells, and subsequently the transformed colonies were grown overnight at 37 °C on LB plates containing 25 µg/mL Zeocin. After 24 h, the Zeocin-resistant transformants were inoculated into LB medium containing 25 µg/mL Zeocin and grown overnight at 37 °C and 200 rpm. Upon plasmid purification from an overnight bacterial culture, the presence of the insert was determined by PCR amplification using CatC-specific primers. The expression vector bearing the CatC cDNA, pPICZαA-CatC, was then linearized with *Sac*I to integrate the expression cassette into the genome of *P. pastoris* and electroporated into *P. pastoris* X-33 host cells using standard procedures (Invitrogen). After transformation, aliquots of 100 µL were plated on YPD plates (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) containing 100 µg/mL Zeocin and incubated for 2 days at 30 °C. The presence of the expression cassette in the genome of Zeocin-resistant clones was confirmed by colony PCR using CatC-specific primers.

2.4. Expression and analysis of CatC in *Pichia pastoris*

The clones proven to contain recombinant human CatC integrated into the *P. pastoris* genome were selected and examined for productivity. Briefly, a single recombinant clone was picked and grown in 25 mL of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base, 4 × 10^{-5}% biotin, and 1% glycerol supplemented with 100 µg/mL Zeocin) at 30 °C and 250 rpm until an OD_{600} of

2.0–6.0 was reached. The cells of this preculture were then collected by centrifugation (at room temperature and 4000 × *g* for 5 min) and used to inoculate 100 mL of BMMY medium (same as BMGY but using 0.5% methanol instead of glycerol) in a 1-L baffled flask to an OD_{600} of about 1.0. Induction of the recombinant protein expression was performed and maintained by daily feeding of the culture with methanol (a final concentration of 0.5%). After 72 h, the cells were harvested by centrifugation (at 4 °C and 4000 × *g* for 10 min) and the clear supernatant was concentrated (20-fold) with ultrafiltration and then recovered as a crude enzyme preparation. The resulting supernatant sample was analyzed by SDS-PAGE using polyacrylamide gel (12.5%) with a stacking gel (5%) under reducing conditions.

2.5. Proteolytic maturation and activity studies of CatC

Since *P. pastoris* expression systems lack papain-like cysteine proteases in their genome, papain was used to activate recombinant CatC to mimic the in vivo proteolytic process. First, the concentrated *P. pastoris* culture supernatant containing proCatC was loaded on a HiTrap SP column and eluted with a 0–1 M NaCl gradient in 20 mM citric acid, pH 6.5, containing 1 mM EDTA and 5 mM dithiothreitol to further purify the preparation. The eluted proCatC between 0.2 and 0.3 M NaCl was then treated with papain (20 µg/mL) to digest the CatC precursor in a shaker for 4 h at 37 °C and 200 rpm. Residual CatC activity was monitored in the aliquots withdrawn from the preincubation mixture at predetermined time points. The activities were measured in an assay buffer containing 20 mM citric acid, pH 4.5, 150 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol using the chromogenic substrate Gly-Phe-*p*NA (0.1%), which gives yellow-colored *p*-nitroaniline (*p*-NA) upon hydrolysis, and the change in absorbance at 405 nm (37 °C) was monitored. For calibration, *p*-NA solutions of 1.0 to 50 mM were used. At the end of incubation, proteolytic activation of CatC was analyzed by SDS-PAGE. After activity assays, inhibition studies with iodoacetamide were performed in the assay buffer with increasing concentrations (0.01/0.1/1.0 mM) to examine the inhibition of the produced enzyme. One unit of CatC activity was expressed as the amount of enzyme required to convert 1 µmol of substrate per minute under optimum reaction conditions. Each result represents the mean of three independent experiments performed in triplicate on the same sample.

3. Results

3.1. Synthesis of CatC cDNA and construction and transformation of pPICZαA-CatC vector

A full-length CatC cDNA, corresponding to amino acids 12–475, was synthesized from interleukin-2 stimulated PBMCs. Since human CatC is present at low levels in unstimulated lymphocytes, the transcription of the CatC gene was upregulated by IL-2 before total RNA isolation from PBMCs. After induction of human CatC mRNA for 48 h, total RNA of the stimulated cells was isolated and reverse transcribed into cDNA. The full-length CatC cDNA was then successfully amplified from the cDNA pool using CatC primers and a 1.4-kb fragment encoding CatC was produced (Figure 1).

After gel purification, the concentrated full-length CatC was cloned into the pGEM-T Easy cloning vector and transformed into *E. coli* DH5α cells. The nucleotide sequence of the insert in the selected transformed clones was then determined to confirm that changes had not occurred in the cDNA sequence using the T7 and SP6 promoter primers on a commercial basis.

After confirming the nucleotide sequence of the insert, *Not*I digestion was performed to liberate the 1.4-kb CatC cDNA from the pGEM-T-CatC vector and it was ligated into the *Not*I site at the multicloning site of the pPICZαA expression vector to construct the pPICZαA-CatC vector (Figure 2).

The pPICZαA-CatC expression vector was then linearized with *Sac*I to integrate the expression cassette into the *P. pastoris* genome and transformed into the strain X-33, yielding the new strain *P. pastoris* X-33 pPICZαA-CatC-WT. Genomic integration of the expression cassette encoding CatC was confirmed by colony PCR using CatC primers as shown in Figure 3.

3.2. Expression and activation of recombinant human CatC

To generate a functional protease for in vitro studies, recombinant human cathepsin C production was investigated in the yeast *P. pastoris* expression system. Since this strain does not contain papain-like cysteine proteases in its genome (Linnevers et al., 1997) and the proenzyme is not capable of autoactivation (Dahl et al., 2001), the analysis of the culture supernatant revealed that recombinant human CatC was secreted as a proenzyme with a mass of approximately 58 kDa, which is consistent with the mass of proCatC previously reported (Dahl et al., 2001) (Figure 4).

It was previously demonstrated that maturation of proCatC with papain results in a 2000-fold increase in activity (Lauritzen et al., 1998). On the basis of this observation, proCatC was treated with papain to mimic the in vivo proteolytic process and to exert its proteinase activity. As shown in Figure 5, the inactive proCatC was gradually converted into its active form in the medium. SDS-PAGE analysis of the sample showed that the 58-kDa proCatC band disappeared after the incubation and new protein bands appeared with molecular masses of 25, 18, 12, and 7 kDa, which are in good agreement with the molecular weights obtained in other studies. It has been reported that mature CatC isolated from mammalian

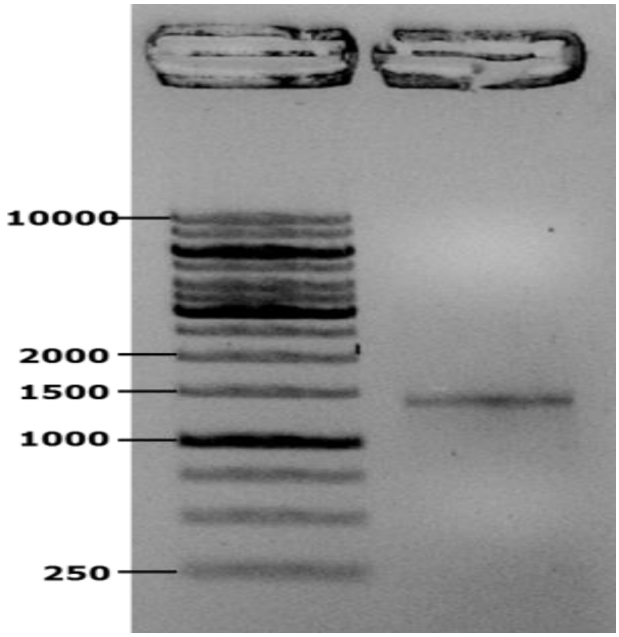

Figure 1. Amplification of 1.4-kb CatC fragment from the cDNA pool using CatC primers.

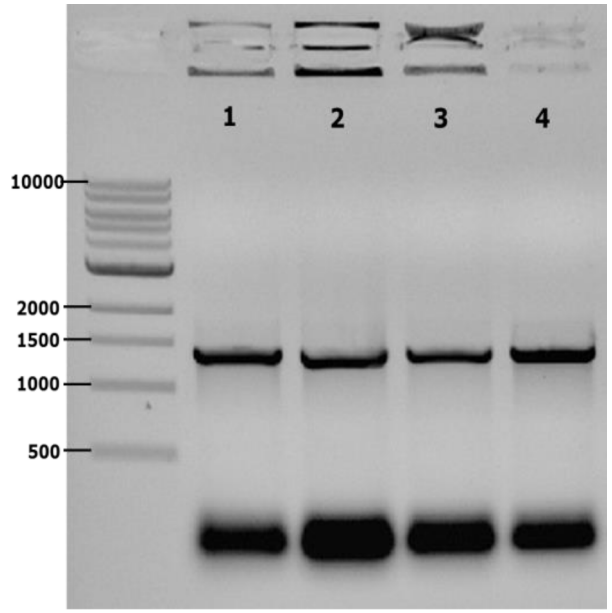

Figure 3. Confirmation of genomic integration of expression cassette encoding CatC by PCR using CatC primers (1–4: PCR using genomic DNA of selected clones).

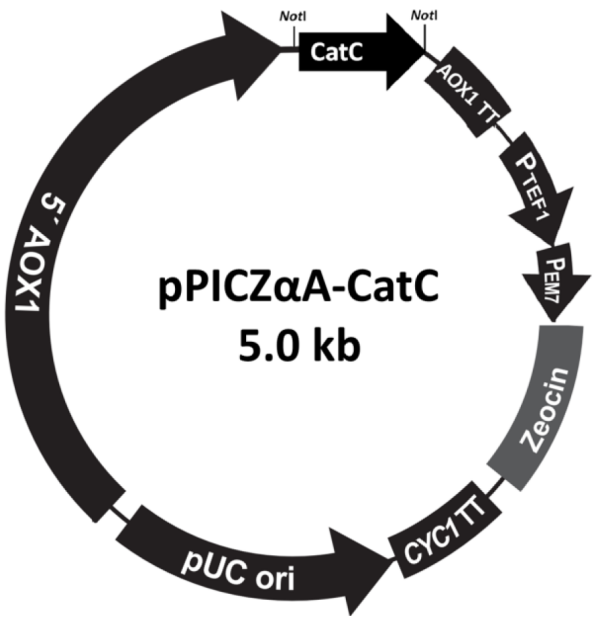

Figure 2. Map of the expression vector pPICZαA-CatC. 5'AOX1: Alcohol oxidase 1 promoter region allows methanol-inducible expression in *P. pastoris*; CatC: sequence encoding human cathepsin C; AOX1 TT: transcription termination region; P_{TEF1}: TEF1 promotor; P_{EM7}: EM7 promotor; Zeocin: Zeocin resistance gene; CYC1 TT: transcription termination region processing of resistance gene mRNA and pUC-ori: origin of replication functional in *E. coli*.

tissues was cleaved into a heavy chain of 25 kDa (19 kDa without glycosylation) and a light chain of 7.8 kDa, and the molecular weights of 18 and 12 kDa correspond to the excised propeptide (Dolenc et al., 1995; Cigic et al., 1998). The two bands at 37 and 28 kDa, corresponding to the intermediate polypeptides, resulted from incomplete proteolytic maturation (Demirov et al., 1999), whereas the 21-kDa band corresponds to papain.

Both autocatalytic and papain-induced activation levels of the wild-type proenzyme into active CatC were monitored. According to the results, autoactivation was very weak and showed no enzymatic activity, whereas incubation of the precursor with papain resulted in activation and processed the proenzyme into its active mature form. After 4 h at 37 °C incubation, the activation level of CatC reached 9.6 U/mg, corresponding to approximately 23 mg of mature recombinant CatC per liter of culture (Figure 6). This enzymatic activity toward the studied synthetic substrate was high and comparable with that of the results reported previously (Dahl et al., 2001; Yang et al., 2011).

Lastly, to determine whether the produced enzyme could be inhibited, an inhibition assay was performed after maturation of CatC. Since CatC does not possess a specific inhibitor, iodoacetamide was used as a general cysteine protease inhibitor. Iodoacetamide inhibited CatC by 6.4%, 22.1%, and 52.7% for 0.01, 0.1, and 1.0 mM concentrations, respectively, confirming that the produced active enzyme could be used for the development of specific inhibitors.

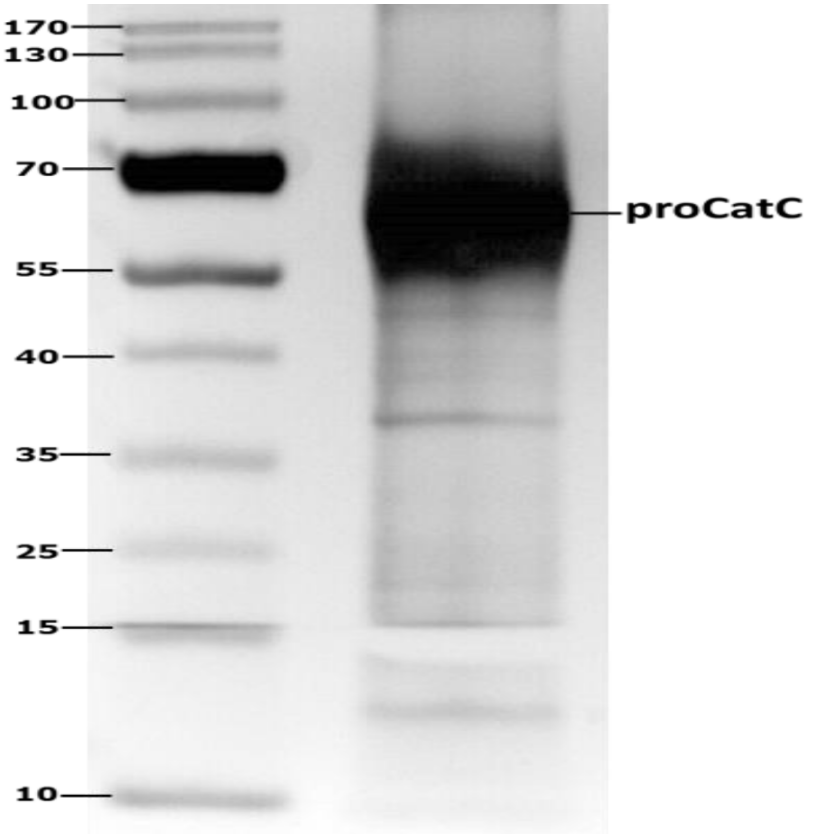

Figure 4. SDS-PAGE analysis of the culture supernatant.

4. Discussion

In this work, the methylotrophic yeast *P. pastoris* expression system was tested for its ability to express human cysteine proteinase CatC. Successful recombinant production of human CatC is reported. Several expression systems were studied in recent years for the recombinant production of human CatC. In those previous reports, recombinant production of CatC was described in mammalian and baculovirus-infected insect cell systems (Dahl et al., 2001; Yang et al., 2011). However, mammalian systems are complicated, expensive to handle, and mostly inappropriate for high-level expression of a functional enzyme. Although baculovirus-infected insect cells are simpler systems as compared to mammalian systems, their complicated cultivation and expensive media makes large-scale production difficult (Demain and Vaishnav, 2009). In contrast to these systems, the yeast *P. pastoris* is a much cheaper alternative that provides ease of handling in transformation, cultivation, and upscaling of the strain (Cregg et al., 1993). The benefits of using this strain are: 1) cells grow easily to high cell densities, 2) the strain allows posttranslational modifications similar to eukaryotic cells, 3) gene expression is strictly controlled by carbon sources and induced by methanol, 4) this protease-deficient strain can increase the yield of secreted recombinant protein by eliminating undesirable proteolysis risk occurring after secretion of the protein, and 5) the strain secretes few native proteins into the culture medium, thereby allowing purification of the secreted recombinant protein in a single step from the culture medium (Linnevers et al., 1997; Cereghino and Cregg, 2000). To date, a number of active human cathepsin proteinases, including cathepsin K (Linnevers et al., 1997), F (Wang et al., 1998), and V (Brömme et al., 1999), have been expressed in *P. pastoris*, whereas there is no information available on human cathepsin C so far.

CatC is an unusual cysteine protease in that it has a very long pro-peptide of ~200 amino acids at the extension of the N-terminus. The residual propart of this proregion remains attached in the mature enzyme, which makes CatC unique, whereas the internal activation peptide is excised during maturation (Cigic and Pain, 1999). In this process, the structure of CatC undergoes a series of transformations from the proenzyme to the mature enzyme (Muno et al., 1993). ProCatC is not capable of autoactivation, whereas many cathepsins of this family such as cathepsins B, K, L, S, F, and V possess this feature (Dahl et al., 2001). The endoproteases that excise the CatC proregion and cleave the catalytic region into heavy and light chains in vivo

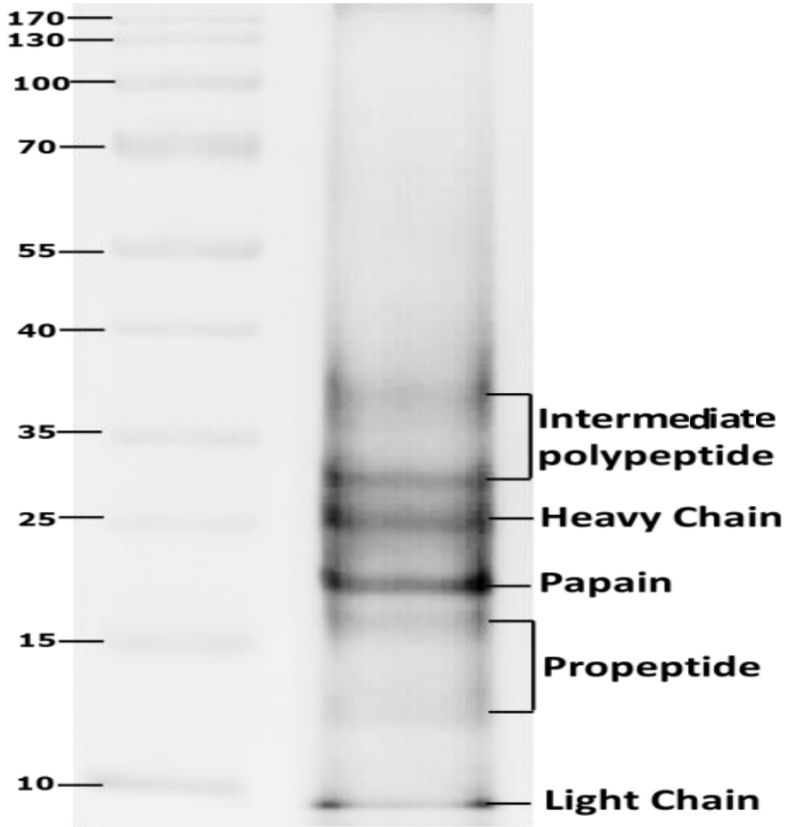

Figure 5. SDS-PAGE analysis of proCatC maturation by papain. Purified proCatC was incubated with papain (20 µg/mL) at pH 6.5 and 37 °C for 4 h. The reaction was stopped by the addition of SDS-PAGE sample buffer.

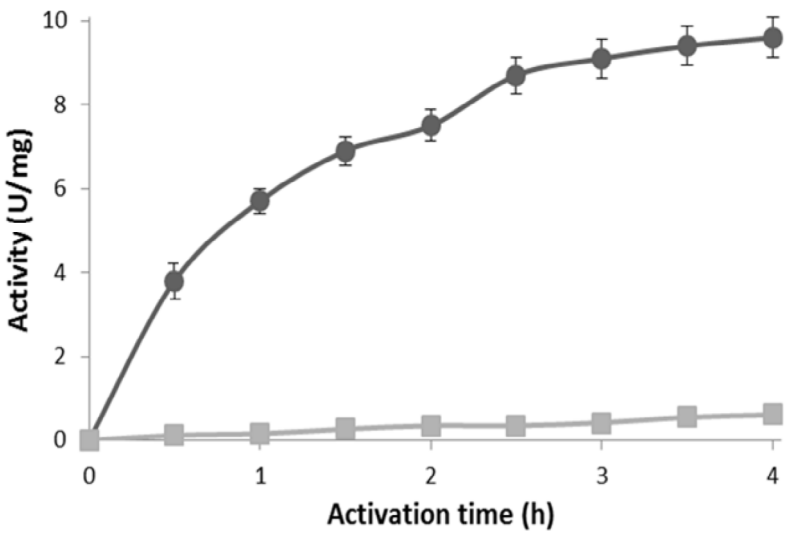

Figure 6. Time-dependent activation of CatC during maturation of proCatC at pH 6.5, 37 °C with papain (●) or at pH 4.5, 37 °C without (▪) (to examine autoactivation). Aliquots were withdrawn from the preincubation mixture at the indicated time points, and the activity was monitored in assay buffer containing 20 mM citric acid, pH 4.5, 150 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol with 0.1% Gly-Phe-*p*NA.

are unknown, but it has been shown that proCatC can be efficiently activated with papain, and cathepsin L and S (Lauritzen et al., 1998; Dahl et al., 2001). Therefore, proCatC was treated with papain to mimic the in vivo proteolytic process and activate the proenzyme into its mature form, which led to active recombinant human CatC being successfully obtained. Mature CatC reached high specific activity (U/mg) in 4 h towards synthetic substrate Gly-Phe-*p*NA and was considerably inhibited after in vitro incubation with the general cysteine protease inhibitor iodoacetamide, which corroborates the use of recombinant CatC in the development of specific inhibitors against CatC-related diseases. Furthermore, recombinant CatC could be of potential value for industrial applications as a processing exopeptidase to use in amino acid sequencing of proteins and removal of fusion peptides from the amino termini of recombinant proteins.

In conclusion, this study demonstrated for the first time that the yeast *P. pastoris* is a functional heterologous protein expression host for the production of recombinant human CatC and may serve as a faster and cheaper alternative to other expression systems, which might promote future studies on the development of specific CatC inhibitors and the use of CatC as a processing enzyme in the pharmaceutical and biotechnology industries.

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