Acta Biochim Biophys Sin 2011, 43: 568–575 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr047.
Advance Access Publication 9 June 2011

Original Article

Purification, cDNA cloning, and recombinant expression of chymotrypsin C from porcine pancreas

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Chymotrypsin C is a bifunctional secretory-type serine protease in pancreas; besides proteolytical activity, it also exhibits a calcium-decreasing activity in serum. In this study, we purified activated chymotrypsin C from porcine pancreas, and identified its three active forms. Active chymotrypsin C was found to be different in the length of its 13-residue activation peptide due to carboxydipeptidase (present in the pancreas) degradation or autolysis of the activated chymotrypsin C itself, resulting in the removal of several C-terminus residues from the activation peptide. After limited chymotrypsin C cleavage with endopeptidase Lys C, several purified peptides were partially sequenced, and the entire cDNA sequence for porcine chymotrypsin C was cloned. Recombinant chymotrypsinogen C was successfully expressed in *Escherichia coli* cells as inclusion bodies. After refolding and activation with trypsin, the comparison of the recombinant chymotrypsin C with the natural form showed that their proteolytic and calcium-decreasing activities were at the same level. The successful expression of chymotrypsin C gene paves the way to further mutagenic structure–function studies.

Keywords porcine pancreas; sequence determination; gene cloning; gene expression

Received: January 28, 2011 Accepted: March 31, 2011

Introduction

Chymotrypsin C, the third member of the chymotrypsin family after chymotrypsin A and B, is first purified and identified by Folk and Schirmer from porcine pancreas [1]. Chymotrypsin C tends to hydrolyze the peptide or ester bond of aromatic or long aliphatic amino acids, particularly at leucine residues [1]. Similar to other family members, chymotrypsin C is secreted as an inactive zymogen and activated by trypsin, forming a 13-residue activation peptide that is linked to the major part of enzyme by a disulfide bond [1,2]. In 1992, Tomomura *et al*. [3] purified a serum calcium-decreasing factor, caldecrin, from porcine pancreas, and found that it also exhibited serine protease activity. In addition, caldecrin remained active even when its protease catalytic sites were irreversibly blocked, indicating that these two biological activities are distinct and unrelated [3,4]. Caldecrin also inhibits osteoclast differentiation by suppressing the nuclear factor of activated T cells (NFATC1) [5]. Additional studies have revealed that chymotrypsin C and caldecrin are actually the same protein. Chymotrypsin C also plays a role in regulating trypsin activation by selectively and rapidly cleaving the Leu81-Glu82 peptide bond of trypsinogen in a Ca^{2+} concentration-dependent manner [6,7]. Genetic studies have further shown that an inherited chymotrypsin C mutation (low activity/secretion) inevitably leads to chronic pancreatitis [8,9], and that chymotrypsin C is down-regulated in pancreatitis [10]. These studies all suggested that chymotrypsin C should not simply be regarded as a digestive enzyme, and it also plays an important regulatory role in many physiological functions *in vivo*.

Studies on cDNA of human and rat chymotrypsinogen C have revealed that their predicted sequences are rather conserved, comprising a signal peptide of 16 residues and a mature form of 252 residues that includes 10 cysteine residues [11,12]. The zymogen form is activated by trypsin and cleaved into a small and a large peptide fragment linked by a disulfide bond [2]. To date, only the crystal structure of bovine ternary complex, including the zymogens of chymotrypsin C, metalloexopeptidase, and proteinase E has been resolved [13].

In this study, we purified activated chymotrypsin C from porcine pancreas. Based on the partially determined peptide sequences, the entire cDNA sequence of porcine chymotrypsinogen C was cloned, and successfully expressed in *Escherichia coli* cells. It was found that after refolding and activation with trypsin, the recombinant chymotrypsin C

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exhibited the same serine proteolysis activity and serum calcium-decreasing activity as the natural one. Thus, our findings pave a way to further structure−function studies of chymotrypsin C in different physiological roles by mutagenesis.

Materials and Methods

Purification of chymotrypsin C from porcine pancreas

The porcine pancreas acetone powder was provided by the biochemical company (Deyang Pharmaceutical Bioindustry, Deyang, China), which was used for kallikrein production. An appropriate amount of soybean trypsin inhibitor was added to the acetone powder to avoid excessive activation of trypsinogen, which would result in autolysis of many proteases activated by trypsin. Ten grams of porcine pancreas acetone powder was extracted with 500 ml cold extraction buffer (50 mM CH_3COONH_4, pH 5.0) for 10 min. After centrifugation at 5000 *g* for 10 min, the supernatant was collected and loaded onto a diethylaminoethylcellulose (DEAE) Sepharose CL 6B (Pharmacia, Uppsala, Sweden) ion-exchange column (4 × 15 cm). The low pH buffer was used to facilitate the removal of most of the contaminating proteins in the starting buffer. The column was then eluted by stepwise increments of NaCl from 0.1 to 0.2 M. The chymotrypsin C fraction was eluted at 0.2 M NaCl, collected, and dialyzed against distilled water. The amount of lyophilized protein obtained was ∼260 mg. A reverse-phase high-performance liquid chromatography (HPLC) column (Agilent 300SB-C8, 4.6 × 250 mm; Agilent, Santa Clara, USA) was used for further purification.

Protease activity assay

The proteolytic activity of natural or recombinant chymotrypsin C was measured by the hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as described previously [14]. The final concentration of BTEE was 5×10^{-4} M. The reaction buffer was 0.1 M Tris buffer (pH 7.8) containing with 0.05 M $CaCl_2$.

Limited proteolysis of chymotrypsin C with endopeptidase Lys-C

The purified chymotrypsin C around 2 mg was dissolved in 5 ml of 0.1 M Tris-HCl and 1 mM EDTA, pH 8.0, and dithiothreitol stock solution was added to a final concentration of 10 mM. The reduction reaction was carried out at 37°C for 2 h. Following the reduction step, iodoacetic acid stock solution was added to a final concentration of 100 mM. The reaction mixture was stored in the dark for 0.5 h at 37°C, and the reaction was stopped by adding 250 mM trifluoroacetic acid (TFA). The reduced and carboxymethylated portion was obtained by dialyzing against distilled water, and was then lyophilized. The lyophilized sample was dissolved in cleavage buffer (50 mM NH_4HCO_3 and 2 M urea), to which lysyl-endopeptidase Lys C was added to a final ratio 100:1. This mixture was incubated at 37°C for 9 h and then lyophilized. The lyophilized sample was dissolved in 0.1%TFA and applied to a reverse-phase HPLC column (Agilent 300SB-C18, 4.6 × 250 mm; Agilent).

Mass spectrometry and peptide sequencing

The molecular masses of proteins were measured by Q-trap mass spectrometry (Applied Biosystems, Foster City, USA). The amino acid sequences were analyzed by automated Edman degradation (ABI Model 491A; Applied Biosystems, Foster City, USA).

cDNA cloning of chymotrypsin C

Total RNA was extracted from porcine pancreas using TRIZOL Reagent (Invitrogen, Carlsbad, USA). Reverse transcription was carried out using a 3′-RACE kit (Takara, Dalian, China), which contained a 3′-RACE adapter.

For 3′-RACE, a degenerate oligonucleotide primer P1 (5′-AARCCNACNGTNTTYACNCGN-3′, where Y = C or T, and N = G, A, T, or C) was designed based on the partially determined peptide sequence (KPTVFTR) of the purified peptide cleaved from chymotrypsin C. The reverse primer P2 (5′-TACCGTCGTTCCACTAGTGATTT-3′), a 3′-RACE outer primer, matched the complementary strand of the adapter primer used for reverse transcription. The polymerase chain reaction (PCR) conditions were as follows: 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. The amplified ∼300-bp fragment was purified and ligated into pGEM-T Easy vector (Promega, Wisconsin, USA) and then sequenced.

Based on the partial cDNA sequence determined by 3′-RACE, a 5′-RACE kit (Invitrogen, Carlsbad, USA) was used to perform 5′-RACE. The gene-specific primer P3 (5′-TTGGTCTTGACTTCCAAGGCTG-3′) was designed for reverse transcription based on the cDNA sequence downstream from the TGA stop codon. The product of reverse transcription was used as a template for gene-specific PCR amplification. The forward primer P4 (5′-GGCCACGCGTC GACTAGTACGGGGGGGGGGGGGGG-3′), a 5′-RACE abridged anchor primer, was paired with the reverse primer P5 (5′-CAGCTGGATTTTCTGGTCGAT-3′), which was designed based on the determined sequence. The PCR amplification product was purified, ligated into pGEM-T Easy vector, (Promega), and sequenced.

Expression, refolding, and purification of recombinant chymotrypsinogen C in *E. coli*

To express porcine chymotrypsinogen C in *E. coli* cells, chymotrypsinogen C cDNA was inserted into the PET vector (Transgen, Beijing, China). The encoding DNA fragment was confirmed by sequencing. The Transetta

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(DE3)-harboring plasmid was grown overnight at 37°C in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin. A 5-ml aliquot of the overnight culture was added to 500 ml LB medium containing 50 μg/ml of ampicillin. Protein expression was induced by adding 0.5 mM isopropylthio-β-galactoside (IPTG) for 5 h at 37°C, when the cells reach the exponential growth [optical density $(OD)_{600}$= 0.5].

The *E. coli* cells were harvested by centrifugation (5000 *g*, 10 min) and lysed by sonication in 50 ml lysis buffer (0.1 M Tris-HCl, pH 8.0) with 1 mM phenylmethylsulfonyl fluoride After centrifugation (10,000 *g*, 15 min), the pelleted inclusion bodies were washed twice by resuspending the pellet in cell lysis buffer supplemented with 10% Triton X-100. A final wash was performed without 10% Triton X-100. The purified inclusion bodies were then dissolved and denatured in 10 ml lysis buffer containing 4 M guanidine hydrochloride and 5 mM dithiothreitol at room temperature for 6 h (the concentration of chymotrypsinogen C was ~1.5 mg/ml).

The expressed and denatured chymotrypsinogen C was slowly diluted with 1000 ml of refolding buffer (0.1 M Tris-HCl, pH 8.0, 1 M guanidine hydrochloride, 1 mM GSSG, and 2 mM GSH) and stirred at 4°C overnight. The solution was filtered through a 0.22 μm membrane and loaded onto a Ni^{2+} column pre-equilibrated with the washing buffer (0.1 M Tris-HCl, pH 8.0). Chymotrypsinogen C was eluted from the column with 200 mM imidazole.

Results and Discussion

Purification and identification of porcine chymotrypsin C

Taking advantage of the low pI of chymotrypsin C, the extract from acetone powder of porcine pancreas was loaded onto a DEAE ion-exchange column at pH 5.0. Under these conditions, most of the contaminated proteins were washed out; chymotrypsin C could be eluted by using a buffer containing 0.2 M NaCl [**Fig. 1(A)**]. After the final purification step with HPLC, natural chymotrypsin C was obtained in homogeneity. Mass analysis revealed that there were three fractions with molecular weights of 26900, 27011, and 27096, respectively [**Fig. 1(B)**]. Based on the deduced cDNA sequence of chymotrypsinogen C (**Fig. 2**), the molecular weight of activated chymotrypsin was calculated to be ~27325.7. After trypsin activation at the Arg13-Val14 cleavage site, chymotrypsin C was converted to two peptide fragments connected by a disulfide bond. Some small peptides were removed from the C-terminal Arg-13 of the activation peptide [2]. On the basis of the determined molecular weights, these small peptides corresponded to the tetrapeptide (Leu-Ser-Ala-Arg, MW 427.5), the tripeptide (Ser-Ala-Arg, MW 314.4), and the dipeptide (Ala-Arg, MW 227.3) which have been cleaved off from the activation peptide. Their calculated respective molecular weights of the activated chymotrypsin C (26898.2, 27011.4, and 27098.5) were very close to the above-mentioned determined molecular weights. The results indicated that the purified chymotrypsin C was in an activated form, and that its main components were the derivatives from which a tetrapeptide or dipeptide was removed from the C-terminal of the activation peptide, probably by carboxydipeptidase, which has been reported to be present in the pancreas [15,16]. The enzyme could not remove an additional dipeptide, since the subsequent cleavage site, Pro7-Pro8, is resistant to the enzyme. Whereas chymotrypsin C with a tripeptide removed from the activation peptide seemed to be delivered by autolysis of the activated chymotrypsin C itself, it actually tends to cleave the peptide bond from the Leu residue (Leu10–Ser11). Most probably,

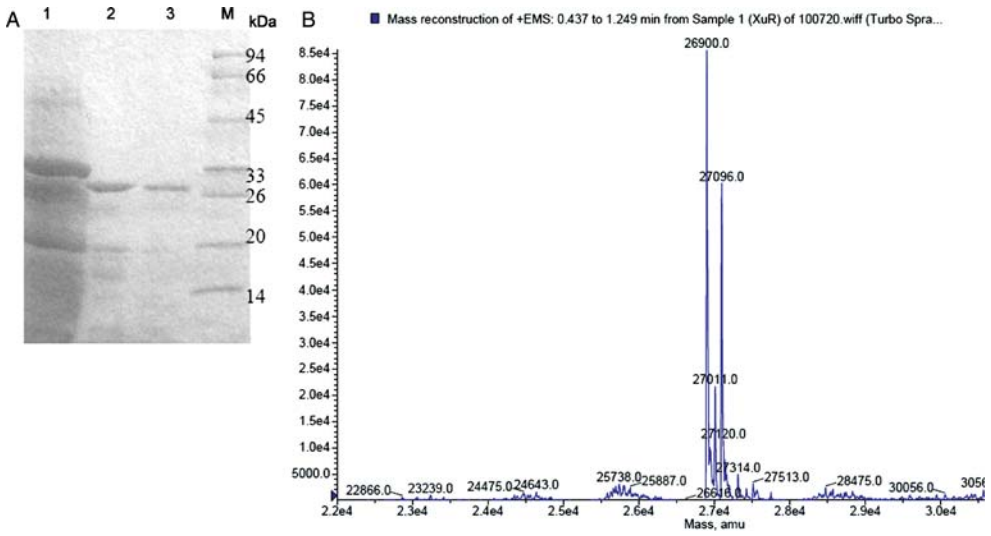

Figure 1 SDS-PAGE (A) and mass spectrogram (B) of chymotrypsin C purified from porcine pancreas (A) Lane 1, crude porcine acetone powder extract loaded on a DEAE column; lane 2, fraction eluted with buffer containing 0.2 M NaCl; lane 3, fraction after further purification by HPLC.

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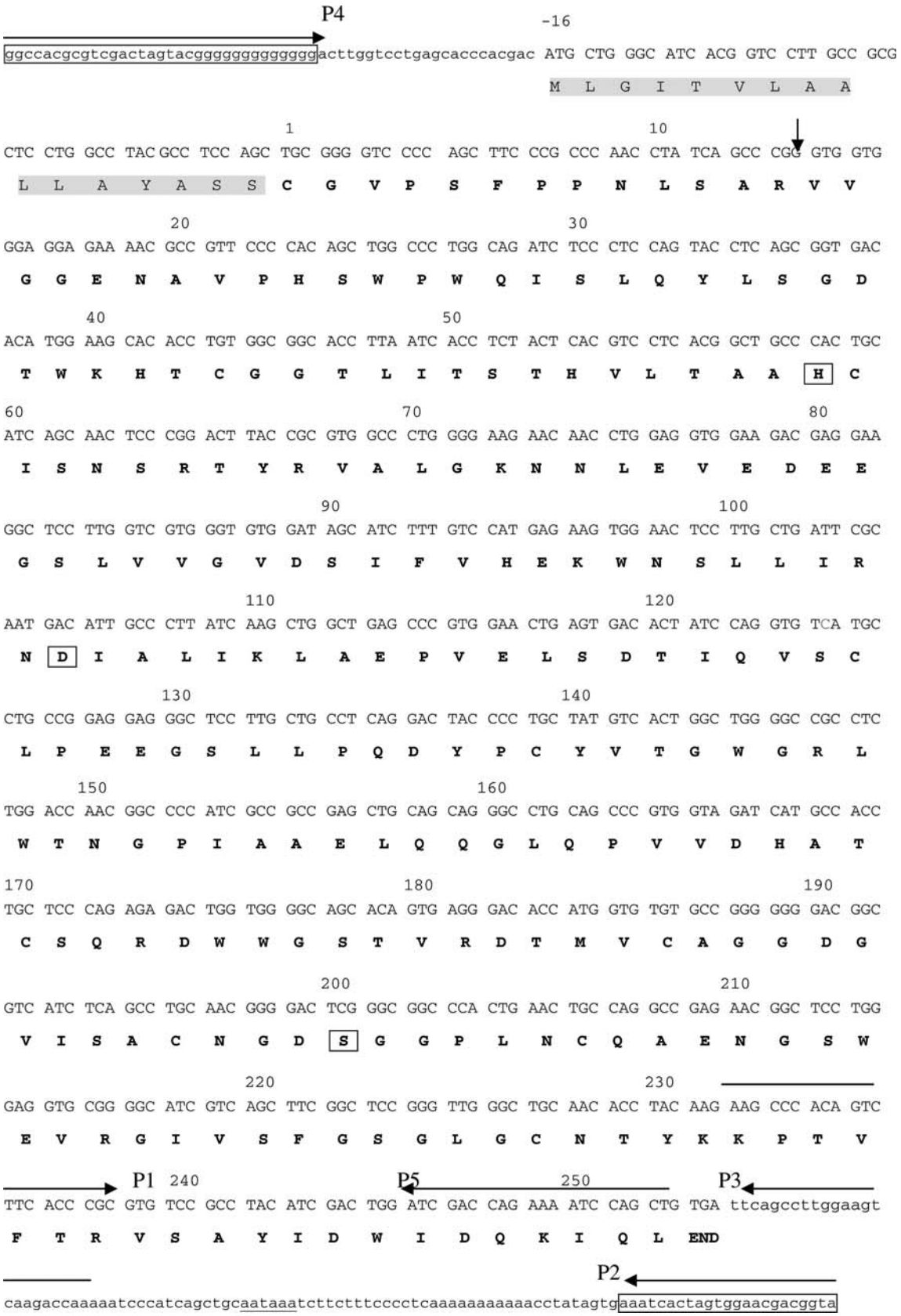

Figure 2 Deduced amino acid sequences and cDNA of porcine chymotrypsinogen C The mature peptide sequence is shown in bold and the polyadenylation signal is underlined. The arrowhead indicates the proteolytic cleavage site and a box surrounds the catalytic triad residues. Nucleotides in the untranslated region are shown as lower case letters. The degenerate primers used for cDNA cloning are indicated by arrows. The nucleotides in the untranslated region belonging to primer 2 and primer 4 are boxed.

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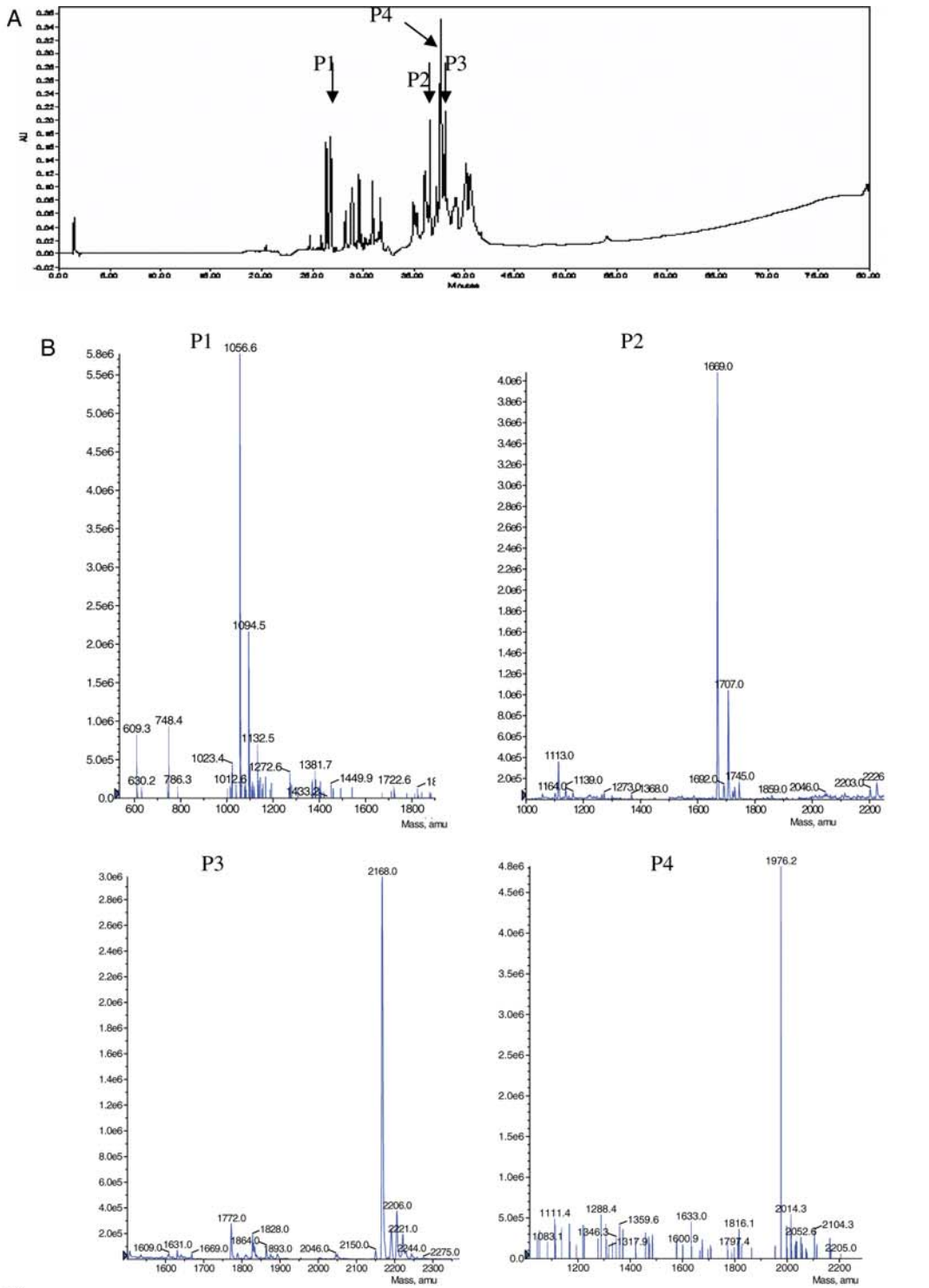

C

Peak number	Determined peptide sequence	Deduced cDNA sequence	Determined M.W.(Da)	Calculated M.W. (Da)
P1	GSGLGCNTY…	GSGLGCNTYK	1056.6	1057.1
P2	WNSLLIRNDIALI…	WNSLLIRNDIALIK	1669.3	1669.0
P3	KPTVFTRVSAYID…	KPTVFTRVSAYIDWIDQK	2167.4	2167.5
P4	VVGGENAVPH……	VVGGENAVPHSWPWQISL	1976.2	1976.2

Figure 3 Separation of chymotrypsin C peptide fragments degraded with Lys-C on HPLC (A), by mass spectrogram analysis (B), and by protein sequencing (C)

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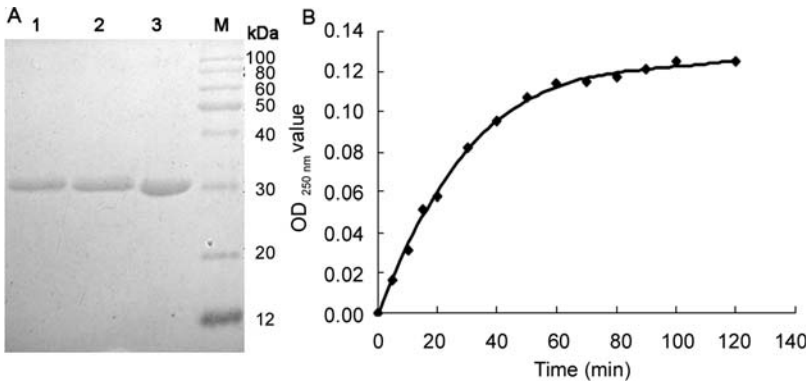

Figure 4 Characterization of the recombinant chymotrypsinogen C (A) SDS-PAGE. Lane 1, recombinant chymotrypsinogen C; lane 2, activated chymotrypsin C after activation with trypsin; lane 3, the natural-activated chymotrypsin C. (B) The activation curve of the purified recombinant chymotrypsinogen C activated by trypsin at a ratio of 1:100. The conditions for active curve determination were as follows: During the different activation time as expressed in *x*-axis, about 2 μg chymotrypsinogen C was added to 1 ml of the reaction buffer with BTEE, the reaction time was 3 min, and the OD increase was then measured and expressed in *y*-axis.

the short 13-residue activation peptide of the activated chymotrypsin C is exposed at the molecular surface, and is thereby liable to be attacked by proteases. It has been reported that the C-terminal three or four residues of the activation peptide are cleaved off from the purified activated chymotrypsin C [17,18].

The amino acid sequence of the purified activated chymotrypsin C was determined. As expected, two residues simultaneously appeared during each Edman degradation step. Residues CGVPSFPP . . . and VVGGENAVPH . . . correspond exactly to the N-terminal sequence of the activation peptide and the remaining part of the activated chymotrypsin C, respectively (**Fig. 2**).

cDNA cloning of chymotrypsin C

In order to obtain the amino acid sequence necessary to clone the cDNA of chymotrypsinogen C, the purified protein was cleaved with an endopeptidase Lys-C, which preferentially cleaves peptide bonds at lysine residues. After enzyme degradation, the resulting peptide fragments of the purified chymotrypsin C were separated by C18 reverse-phase HPLC. The four major peptides purified were analyzed by mass spectrum and protein sequencing. As shown in **Fig. 3**, all determined partial sequences and molecular weights of the four peptides were identical to those of the corresponding fragments later deduced from the cDNA sequence of porcine chymotrypsinogen C (**Fig. 2**).

On the basis of the known sequence KPTVFTR of peak 3, the degenerate primer P1 was designed and paired with the adapter primer P2 for 3′-RACE. For 5′-RACE reverse transcription, the gene-specific primer P3 was designed and paired with the abridged anchor primer P4. The product was then used as a template for gene-specific PCR amplification with primers P4 and P5. Combining the results of 3′-RACE and 5′-RACE, the entire cDNA sequence of porcine chymotrypsin C was determined as shown in **Fig. 2**, the accession number is JF429833.

Porcine chymotrypsinogen C cDNA has an 807-bp open reading frame (**Fig. 2**). A polyadenylation signal, AATAAA, and a poly (A) tail were found in the 3′ untranslated region of the cDNA. The deduced amino acid sequence of chymotrypsinogen C comprises 268 amino acids, including 16 signal peptide residues and 252 mature protein residues. There are 10 cysteine residues that form five disulfide bonds. The catalytic triad is composed of His58, Asp105, and Ser200 residues, which correspond to the His57, Asp102, and Ser195 of chymotrypsin A. Comparing the cDNA sequence that we identified with that of genomic sequence (accession number: XM_003127629) published recently in Nov 2010, the result shows that there is only one base difference, namely, in codon 21 GTT was changed to ATT, the deduced residue Val corresponded to Ile.

Porcine chymotrypsin C exhibits high-sequence similarity about 87% with chymotrypsin C from human, bovine, and rat pancreas [11,12]. As there are no data about the sequence of porcine chymotrypsin A in GenBank, we can only compare the sequences of bovine chymotrypsin A, B with bovine chymotrypsin C. The similarity of chymotrypsin A and B to C both are about 41%.

Expression, refolding, and purification of recombinant chymotrypsinogen C in *E. coli*

In order to obtain the recombinant chymotrypsin C for further structure−function study, we selected *E. coli* expression system. The entire cDNA of chymotrypsinogen C was inserted into a pET vector (Transgene, Beijing, China), with an additional five residues (MELAL) at the N-terminal and a His-tag (KGQFLEHHHHHH) at the

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C-terminal. The recombinant vector construct was then expressed in the Rosseta 2(DE3) *E. coli* strain, which contains additional tRNAs that code for rare *E. coli* codons. Recombinant chymotrypsinogen C was successfully expressed in *E. coli* cells in the form of inclusion bodies. After denaturation and refolding, the soluble protein was purified by use of Ni^{2+} column, dialyzed, and lyophilized. The yield of recombinant chymotrypsinogen C from 1 L of LB medium was ~2 mg.

Using a rather complex insect cell system, Tomomura *et al*. have successfully expressed human and rat recombinant chymotrypsinogen C [11,12]. Furthermore, they utilized a bacterial system to express peptide fragments of chymotrypsin C fused with histidine-tagged thioredoxin, which was not removed from the recombinant product [19]. In the present study, the entire chymotrypsinogen C was readily expressed in *E. coli* system and the recombinant product was successfully refolded and purified, paving the way toward further mutagenesis-based structure−function studies of chymotrypsin C.

Characterization of the recombinant chymotrypsinogen C

The N-terminal sequence and molecular weight of recombinant chymotrypsinogen C were determined. After each Edman degradation reaction, only one residue appeared; the first five residues were MELAL, which came from the pET expression vector, and were followed by CGVPSFP . . . , namely, the N-terminal sequence of chymotrypsinogen C. The determined molecular weight was 29382.0, which is close to the calculated molecular weight of 29391.1, including the five additional N-terminal residues and the C-terminal His-tag. These findings indicate that the recombinant protein was a zymogen and was purified in homogeneity.

The purified recombinant chymotrypsinogen C was then activated by trypsin. The weight ratio of zymogen to trypsin was 100:1, and the substrate BTEE was used to monitor the generated activity. The sodium dodecyl sulphate-polyacrylamide gel profile of the recombinant zymogen, the activated enzyme as well as the natural activated chymotrypsin C is shown in **Fig. 4(A)**. The activation time course showed that the zymogen followed a typical first-order reaction [**Fig. 4(B)**].

The recombinant-activated chymotrypsin C was composed of two major fractions due to chymotrypsin C autolysis itself. The fraction that had a determined molecular weight of 27897.0 corresponded to the recombinant-activated chymotrypsin C, from which the additional N-terminal MELAL fragment and the partial C-terminal EHHHHHH of the His-tag KGQFLEHHHHHH were cleaved off, and its calculated molecular weight was 27899.4. The other fraction had a determined molecular weight of 27571.0 and corresponded to the recombinant-activated chymotrypsin C, in which the tripeptide SAR following the Leu reside was further cleaved off similar to the natural-activated chymotrypsin C, and its calculated molecular weight was 27585.1. All the cleavage sites occurred at Leu residues, which are preferentially hydrolyzed by chymotrypsin C itself [1].

Activation of chymotrypsinogen C appears to depend primarily on the Arg13-Val14 bond to release the 13-residue activation peptide, regardless of whether several small peptides are further cleaved off from the activation peptide by the activated chymotrypsin C itself or by carboxydipeptidase. The relaxation of the activation peptide would cause a conformational change of the zymogen, which would then convert it to an active enzyme such as in the case of chymotrypsinogen A activation [20].

The proteolytic and serum calcium-decreasing activities of purified recombinant chymotrypsin C were also measured and compared with those of the natural enzyme; their levels were, as expected, nearly identical (data not shown).

Funding

This work is supported by a grant from the Special Fund of State Key Laboratory of Molecular Biology.

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