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• MAST CELL AND INFLAMMATORY BOWEL DISEASE •

Cloning and expression of human colon mast cell carboxypeptidase

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Abstract

AIM: To clone and express the human colon mast cell carboxypeptidase (MC-CP) gene.

METHODS: Total RNA was extracted from colon tissue, and the cDNA encoding human colon mast cell carboxypeptidase was amplified by reverse-transcription PCR (RT-PCR). The product cDNA was subcloned into the prokaryotic expression vector pMAL-c2x and eukaryotic expression vector pPIC9K to construct prokaryotic expression vector pMAL/human MC-CP (hMC-CP) and eukaryotic pPIC9K/hMC-CP. The recombinant fusion protein expressed in *E.coli* was induced with IPTG and purified by amylose affinity chromatography. After digestion with factor Xa, recombinant hMC-CP was purified by heparin agarose chromatography. The recombinant hMC-CP expressed in *Pichia pastoris* (*P.pastoris*) was induced with methanol and analyzed by SDS-PAGE, Western blot, N-terminal amino acid sequencing and enzyme assay.

RESULTS: The cDNA encoding the human colon mast cell carboxypeptidase was cloned, which had five nucleotide variations compared with skin MC-CP cDNA. The recombinant hMC-CP protein expressed in *E.coli* was purified with amylose affinity chromatography and heparin agarose chromatography. SDS-PAGE and Western blot analysis showed that the recombinant protein expressed by *E. coli* had a molecular weight of 36 kDa and reacted to the anti-native hMC-CP monoclonal antibody (CA5). The N-terminal amino acid sequence confirmed further the product was hMC-CP. *E. coli* generated hMC-CP showed a very low level of enzymatic activity, but *P. pastoris* produced hMC-CP had a relatively high enzymatic activity towards a synthetic substrate hippuryl-L-phenylalanine.

CONCLUSION: The cDNA encoding human colon mast cell carboxypeptidase can be successfully cloned and expressed in *E.coli* and *P. pastoris*, which will contribute greatly to the functional study on hMC-CP.

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INTRODUCTION

Mast cells and their inflammatory mediators have been implicated to play a pivotal role in intestinal diseases such as inflammatory bowel disease[1-7], collagenous colitis[8,9], intestinal anaphylaxis[10,11] and irritable bowel syndrome[11,12]. Mast cell neutral proteases constitute more than 50% granule proteins in mast cells. They are tryptase, MC-CP, chymase, and a cathepsin G-like protease[13-16]. Upon degranulation, these neutral proteases are released and carry out numerous functions in tissues nearby or distant as pro-inflammatory mediators. Recently it was found that mast cell products tryptase and histamine might play an important role in the amplification of degranulation signals in human[17-21].

hMC-CP is a distinctive carboxypeptidase, which is exclusively located in MC_{TC} mast cells, possesses pancreatic carboxypeptidase A (CPA)-like activity, but has a closer amino acid sequence identical to carboxypeptidase B (CPB)[13,22]. The evolution analysis demonstrated that MC-CP originated from a gene duplication along the pancreatic CPB lineage rather than along the pancreatic CPA lineage[22,23]. Although hMC-CP has been detected in skin, lung, and intestinal tissues with immunohistochemistry[13,22,24], and hMC-CP genes from skin and lung were cloned[22,23,25], hMC-CP gene from intestinal tissue is still unknown.

Investigations on the structures and functions of human tryptase and chymase have made impressive progress and a number of potent functions of these two mast cell proteases were found in last decade. These include induction of microvascular leakage in skin of guinea pig[26], stimulation of inflammatory cell accumulation in peritoneum of mouse[27,28] and modulation of mast cell degranulation[29,30], indicating that they may be key mediators of allergic inflammation and promising targets for diagnosis and therapeutic intervention[31-35]. However, little is known about the function of hMC-CP except for its ability to cleave angiotension I[13]. This could be resulted from lack of sufficient amount of hMC-CP. In the current study, a procedure for cloning and expression of hMC-CP was developed and enzymatically active human intestinal recombinant MC-CP was produced.

MATERIALS AND METHODS

Materials

RNA extract kit, total RNA purification system, multi-copy *Pichia* expression kit were purchased from Invitrogen (Carlsbad, CA, USA). First strand cDNA synthesis kit, restriction endonucleases, T_4 DNA ligase, the expression vector pMAL-c2x, *E.coli* hosts TB1 and amylose resin were obtained from Biolabs (Beverly, MA,USA). Antibiotics, isopropyl thio-β-D-galctopyranoside (IPTG), heparin agarose, extr-Avidin peroxidase, biotinlated sheep anti-mouse IgG, for *E.coli* growth medium and *P. Pichia* growth medium were from Sigma (Saint Louis, MO, USA). Qiaquick gel extraction kit and *Taq* polymerase were from Qiagen (Hilden, Germany). Protein molecular weight markers were from Bio-Rad (Hercules, CA, USA). A monoclonal antibody against human mast cell carboxypeptidase (CA5) was donated by University of Southampton, UK. All other chemicals were of analytical grade.

Tissue preparation

Human colon tissue was obtained from patients with carcinoma

of colon at colectomy. Only macroscopically normal tissue was used for the study. The specimens were kept in liquid nitrogen until use.

Extraction of RNA

Total cellular RNA was extracted from normal colon tissues according to the manufacturer' s protocol. The purity of RNA was confirmed by formaldehyde denaturing agarose gel electrophoresis, and the concentration of RNA was determined with a spectrophotometer (DU640, Beckman).

Synthsis of cDNA

cDNAs were generated from total RNA by using the ProtoScript™ first strand cDNA synthesis kit. A total of 10 μL of RNA (1 μg), 2 μL of oligo (dT) primer and 4 μL of 2.5 mM dNTP were heated at 70 ℃ for 5 min. Reverse transcription was performed for 1 h at 42 ℃ in a solution (20 μL of total volume) containing 1 μL of 25U/μL M-MuLV reverse transcriptase. The reaction was terminated by incubating the mixture at 95 ℃ for 5 min, and placed on ice immediately.

PCR amplification and cloning of cDNAs

Based on the published DNA sequence of human skin mast cell carboxypeptidase[22], a pair of primers (P1: 5' -GCTATGAGGCTCATCCTGCCTGT-3' ; P2: 5' -GCTTTAGGAAGTATGCTTGAGGATATAC-3') were used to amplify hMC-CP cDNA. A hot-star PCR protocol was followed under the condition: at 95 ℃ for 15 min prior to amplification, then at 94 ℃ for 30 s, at 57 ℃ for 30 s, and at 72 ℃ for 1 min. The amplification was carried out for 30 cycles, followed by incubation at 72 ℃ for 10 min. The PCR products were analyzed with 1% agarose gel electrophoresis, and recovered with a Qiaquick gel extraction kit. The purified PCR product was cloned to pGEM-T Easy vector, forming a new plasmid pGEM/hMC-CP. The ligation mixtures were transformed into *E.coli* Dh5α. The positive recombinant clones were seeded on LB/agar plates with 100 μg/mL ampicillin, and the clones were further determined by PCR and DNA sequencing using a DNA sequencer (ABI 377 PRISM).

Construction of expression vector

To express hMC-CP in *E. coli*, an expression plasmid comprising the expression vector pMAL-C2x and *hMC-CP* cDNA was constructed. For this construction, a pair of specific primers (P3: 5' -GCTGAATTCATCGAGGGAAGGATCCCAGGC AGGCACAGCTAC-3' ; P4: -GCTCTGCAGTTAGGAAGT ATGCTTGAGGATATAC-3') were designed and used to amplify the coding region of the mature hMC-CP. The forward primer contained the recognition sequences for *Eco*R I, coding sequences for Factor Xa rEcognition sequence and the N-terminal region of the mature hMC-CP, and the reverse primer contained the rEcognition sequences for *Pst* I, and the coding sequence of the C-terminal region of the mature hMC-CP. pGEM/hMC-CP was used as the template for PCR. The resulting PCR fragments and pMAL-c2x plasmid were digested with *Eco*R I and *Pst* I. The fragments of interests were recovered from agarose gel, purified and ligated by T4 DNA ligase, which resulted in the expression plasmid pMAL/hMC-CP. The ligation mixtures were used to transform *E.coli* TB1 cells. The positive recombinant products were selected on LB agar plates with 100 μg/mL ampicillin, and confirmed by PCR and DNA sequencing.

To express hMC-CP in pichia pastoris, aNother expression plasmid comprising the expression vector pPIC9K and hMC-CP cDNA was constructed. For this construction, a pair of specific primers (P5: 5' -GCTGAATTCATCCCAGGCAG GCACAGCTAC-3' ; P6: -TACGCGGCCGCTTAGGAAG TATGCTTGAGGATATAC-3') were designed and used to amplify the coding region of the mature hMC-CP. The forward primer contained the recognition sequences for *Eco*R I, and the reverse primer contained the recognition sequences for *Pst* I. pGEM/hMC-CP was used as the template for PCR. The resulting PCR fragments and pPIC9K plasmid were digested with *Eco*R I and *Not* I. The fragments of interests were recovered from agarose gel, purified and ligated by T4 DNA ligase, which resulted in the expression plasmid pPIC9K/hMC-CP. The ligation mixtures were used to transform *E.coli* DH5α cells. The positive recombinant products were selected on LB agar plates with 100 μg/mL ampicillin. The nucleotide sequences of cDNA insert and flanking sequence were verified. The expression plasmid pPIC9K/hMC-CP was linearized by digestion with *Bgl* II. Competent cells of *P. pastoris* GS115 were prepared for electroporation with the linearized plasmid pPIC9K/hMC-CP. The electroporation was performed in a 2 mm gap cuvette at 2.0 kV, 25 μF, and 200Ω using a gene-pulser (Bio-Rad). Transformants were screened for a His^+ pheNotype on minimal dextrose (MD) agar plates. MD and minimal methanol (MM) plates were used to identify Mut^S clones. YPD plates containing Geneticin at a final concentration of 0.25, 0.5, 0.75, 2.0, 3.0, 4.0 mg/mL were used to screen multiple inserts for further expression.

Expression of recombinant hMC-CP

E.coli TB1 cells harboring the expression plasmid pMAL/hMC-CP were inoculated into LB medium containing 100 μg/mL ampicillin overnight at 37 ℃ in an orbital shaker (220 rpm). IPTG was added to a final concentration of 0.3 mM before the culture mixture was transferred to a 23 ℃ air shaker.

For the expression of hMC-CP in *P. pastoris*, a single colony of GS115 harboring the expression plasmid pPIC9K/hMC-CP was inoculated into 200 mL of buffered minimal glycerol complex medium (BMGY), and grew at 30 ℃ until the culture reached an A_{600}=2.0. Cultured cells were harvested by centrifugation and transferred to 1/10 of the original culture volume of buffered minimal methanol complex medium (BMMY), then grew at 30 ℃. Methanol was added to a final concentration of 0.5% (v/v) every 24 h to maintain induction.

Purification of recombinant hMC-CP

At 24 h after induction, the bacterial cells were harvested by centrifugation at 5 000 g for 10 min at 4 ℃. The pellet was resuspended in 50 mL ice-cold cells lysis buffer (20 mM Tris, 200 mM NaCl, 0.01% Triton X-100) at pH 8.0, then sonicated 6 times for 10 s (300 w) at 30 s intervals. The clarified cell extract was obtained by centrifugation at 20 000 g for 20 min, at 4 ℃.

Amylose resin was used for purification of the fusion protein, and equilibrated with the running buffer (20 mM Tris, 200 mM NaCl, pH8.0), then the cell extract was loaded onto the column at a flow rate. The fusion protein was eluted from column with a buffer containing 10 mM maltose. In order to obtain the recombinant hMC-CP, the fusion protein was cleaved with factor Xa in 20 mM Tris, pH 8.0, containing 100 mM NaCl, 2 mM $CaCl_2$. The digestion was performed at 23 ℃ for 3 h. The above cleavage mixture was applied to heparin agarose in an equilibration buffer (20 mM Tris, 200 mM NaCl, pH8.0), and eluted from heparin agarose by the elution buffer containing 20 mM Tris, 2 M NaCl, pH 8. The fractions containing hMC-CP were collected and stored at -80 ℃. The procedures above were mainly performed at 4 ℃.

SDS-PAGE and Western blotting analysis

SDS-PAGE was performed on a 15% polyacrylamide gel. The gel was then stained with 0.25% Coomassie brilliant blue R-250

or transferred to polyvinylidene fluoride (PVDF) membranes for Western blotting. The membranes were incubated for 1 h at room temperature in PBS containing 4% BSA and 0.02% Tween-20 in order to prevent nonspecific binding. After incubated with CA5, biotinylated sheep anti-mouse antibody followed by extr-avidin peroxidase was added to the strips. The immunoreactive protein was visualized by DAB.

N-terminal amino acid sequence analysis

Protein was sequenced by automated Edman degradation on a model 491A protein sequencer (Applied Biosystem). Purified protein was applied to a SDS-PAGE. After blotting, the polyvinylidene difluoride membranes were stained with Coomassie brilliant blue R-250, the protein bands of interest were cut out for N-terminal amino acids sequence determination.

Protein assay

Protein concentration was determined using the method of Bradford with the protein assay dye reagent concentrator (Bio-Rad) and bovine serum albumin (BSA) was used as a standard protein.

Enzyme activity assay

In this study, the hMC-CP activity was measured spectrophometrically by hydrolyzing a substrate of synthesis peptide of hippuryl-L-phenylalanine[13]. The rate of hydrolysis of hippuryl-L-phenylalanine was determined by measuring the increase in absorbance at 254 nm. The assay mixtures contained 1 mM substrate in 0.05 M Tris-HCl, pH 7.5, 0.5 M NaCl. $\triangle A_{254}$/minute from the initial linear portion of the curve was determined. Unit definition: One unit hydrolyzes one micromole of hippuryl-L-phenylalanine per minute at pH 7.5 and 25 ℃. Bovine pancreatic CPA (51 U/mg, Sigma) was used as positive control.

RESULTS

RT-PCR amplification of hMC-CP cDNA

RT-PCR was performed with total RNA template extracted from human colon tissues. The PCR product showed a single band about 1 250 bp on 1% agarose gel (Figure 1A).

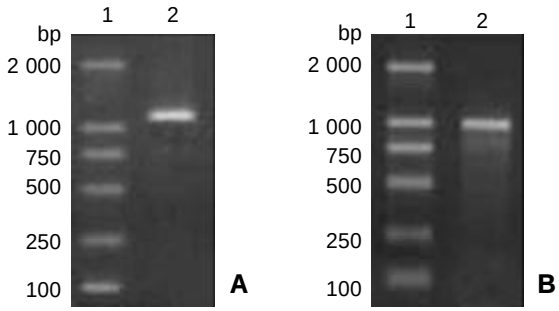

Figure 1 Agarose gel electrophoresis of PCR product. A: hMC-CP cDNA (lane 1: DNA molecular marker; lane 2: hMC-CP cDNA). B: coding region of mature human colon MC-CP cDNA (lane 1: DNA molecular marker; lane 2: PCR product of colon MC-CP cDNA).

DNA sequencing revealed that the human colon MC-CP cDNA had 1 254 bp. The DNA sequence and deduced amino acid sequence of human colon MC-CP are shown in Figure 2. The DNA sequence of human colon MC-CP cDNA was identical to the skin MC-CP except for five nucleotides at the positions 109, 575, 576, 708, 737: C→G, C→T, T→C, A→C, A→T (nucleotide numbering starts from the first codon of mature enzyme). Only the fourth (A1035→C) of the nucleotide differences described above did Not represent an altered amino acid residue in the putative protein products. The other 4

```
1   ATC CCA GGC AGG CAC AGC TAC GCA AAA TAC AAT AAT TGG GAA AAG ATT GTG GCT TGG ACT
    I   P   G   R   H   S   Y   A   K   Y   N   N   W   E   K   I   V   A   W   T
61  GAA AAG ATG ATG GAT AAG TAT CCT GAA ATG GTC TCT CGT ATT AAA ATT GGA TCT ACT GTT
    E   K   M   M   D   K   Y   P   E   M   V   S   R   I   K   I   G   S   T   V
121 GAA GAT AAT CCA CTA TAT GTT CTG AAG ATT GGG GAA AAG AAT GAA AGA AGA AAG GCT ATT
    E   D   N   P   L   Y   V   L   K   I   G   E   K   N   E   R   R   K   A   I
181 TTT ATG GAT TGT GGC ATT CAC GCA CGA GAA TGG GTC TCC CCA GCA TTC TGC CAG TGG TTT
    F   M   D   C   G   I   H   A   R   E   W   V   S   P   A   F   C   Q   W   F
241 GTC TAT CAG GCA ACC AAA ACT TAT GGG AGA AAC AAA ATT ATG ACC AAA CTC TTG GAC CGA
    V   Y   Q   A   T   K   T   Y   G   R   N   K   I   M   T   K   L   L   D   R
301 ATG AAT TTT TAC ATT CTT CCT GTG TTC AAT GTT GAT GGA TAT ATT TGG TCA TGG ACA AAG
    M   N   F   Y   I   L   P   V   F   N   V   D   G   Y   I   W   S   W   T   K
361 AAC CGC ATG TGG AGA AAA AAT CGT TCC AAG AAC CAA AAC TCC AAA TGC ATC GGC ACT GAC
    N   R   M   W   R   K   N   R   S   K   N   Q   N   S   K   C   I   G   T   D
421 CTC AAC AGG AAT TTT AAT GCT TCA TGG AAC TCC ATT CCT AAC ACC AAT GAC CCA TGT GCA
    L   N   R   N   F   N   A   S   W   N   S   I   P   N   T   N   D   P   C   A
481 GAT AAC TAT CGG GGC TCT GCA CCA GAG TCC GAG AAA GAG ACG AAA GCT GTC ACT AAT TTC
    D   N   Y   R   G   S   A   P   E   S   E   K   E   T   K   A   V   T   N   F
541 ATT AGA AGC CAC CTG AAT GAA ATC AAG GTT TAC ATC ACC TTC CAT TCC TAC TCC CAG ATG
    I   R   S   H   L   N   E   I   K   V   Y   I   T   F   H   S   Y   S   Q   M
601 CTA TTG TTT CCC TAT GGA TAT ACA TCA AAA CTG CCA CCT AAC CAT GAG GAC TTG GCC AAA
    L   L   F   P   Y   G   Y   T   S   K   L   P   P   N   H   E   D   L   A   K
661 GTT GCA AAG ATT GGC ACT GAT GTT CTA TCA ACT CGA TAT GAA ACC CGC TAC ATC TAT GGC
    V   A   K   I   G   T   D   V   L   S   T   R   Y   E   T   R   Y   I   Y   G
721 CCA ATA GAA TCA ACA ATT TAC CCG ATA TCA GGT TCT TCT TTA GAC TGG GCT TAT GAC CTG
    P   I   E   S   T   I   Y   P   I   S   G   S   S   L   D   W   A   Y   D   L
781 GGC ATC AAA CAC ACA TTT GCC TTT GAG CTC CGA GAT AAA GGC AAA TTT GGT TTT CTC CTT
    G   I   K   H   T   F   A   F   E   L   R   D   K   G   K   F   G   F   L   L
841 CCA GAA TCC CGG ATA AAG CCA ACG TGC AGA GAG ACC ATG CTA GCT GTC AAA TTT ATT GCC
    P   E   S   R   I   K   P   T   C   R   E   T   M   L   A   V   K   F   I   A
901 AAG TAT ATC CTC AAG CAT ACT TCC TAA
    K   Y   I   L   K   H   T   S  stop
```

Figure 2 Nucleotide sequence and deduced amino acid sequences of mature human colon MC-CP. The nucleotide variations and amino acid substitutions different from the skin MC-CP are underlined and boxed, respectly.

nucleotide variations caused 3 amino acid substitutions at the positions 146, 301, 355. The human colon MC-CP had Gly^{146}, Ile^{301} and Ile^{355} residues, whereas the skin MC-CP had Arg^{146}, Thr^{146} and Asn^{355} residues. In contrast, the colon MC-CP cDNA was 100% identical to the lung MC-CP cDNA.

Construction of expression vector

A 924 bp of PCR product was obtained following amplification of the coding region of the mature hMC-CP (Figure 1B). DNA sequencing showed that the recombinant pMAL/hMC-CP and pPIC9K/hMC-CP plasmids had the correct open reading frame coding for 308 amino acids mature polypeptide and no substitutions were introduced by PCR.

Expression of recombinant hMC-CP in E.coli

As shown in Figure 3A, a high level of expression of an induced protein of about 80 kD was achieved after the *E.coli* harbouring expression plasmid pMAL/hMC-CP, which was in agreement with the expected molecular mass of the fusion protein MBP (45 kDa) and hMC-CP (36 kDa). Figure 3B showed a band at about 80 kDa (expected in *E.coli* cells with IPTG induction) reacted to CA5, suggesting that the recombinant protein had a good immunological activity. The best expression of the recombinant protein after IPTG induction was at 23 ℃ for 16 h (Figure 4). The recombinant products generated by the above procedures were mainly insoluble inclusion body with a small proportion of the soluble recombinant proteins (Figure 5).

In *P. pastoris* expression, 2 colonies resistant to 4.0 mg/ml Geneticin were screened and used for the expression of recombinant protein. There was a substantial quantity of recombinant proteins in cell-free supernatant, and SDS-PAGE showed a major band of approximately 37 kDa (Figure 6A), which reacted to CA5 on Western blot (Figure 6B).

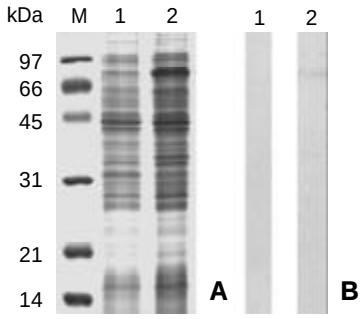

Figure 3 Analysis of recombinant proteins expressed in *E.coli*. A: SDS-PAGE. B: Western blots. M: molecular mass markers; lane 1: without IPTG induction; lane 2: with IPTG induction.

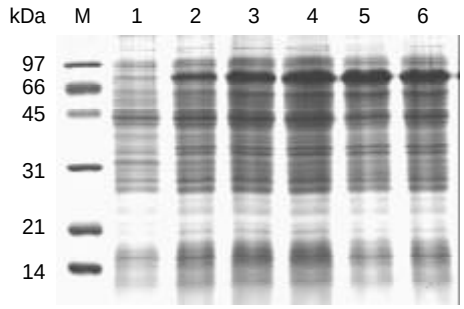

Figure 4 SDS-PAGE analysis of time course of recombinant proteins expressed in *E.coli*. lane 1: before induction; lane 2: 8 h after induction; lane 3: 16 h after induction; lane 4: 24 h after induction; lane 5: 32 h after induction.

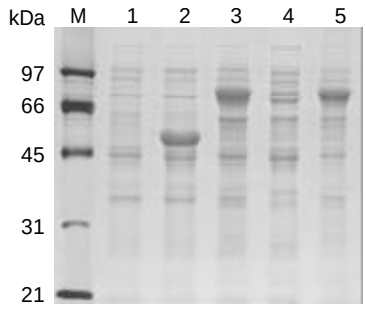

Figure 5 SDS-PAGE analysis of rhMC-CP expressed in *E. coli* TB1 cells. M: molecular weight markers; lane 1: total cellular protein of *E.coli* TB1 cells without IPTG induction; lane 2: total cellular protein of *E.coli* TB1 cells with IPTG induction (control vector); lane 3: total cellular protein of *E.coli* TB1 cells with IPTG induction; lane 4: soluble fraction of cell lysate from *E.coli* TB1 with IPTG induction; lane 5: precipitated fraction of cell lysate from *E.coli* TB1 with IPTG induction.

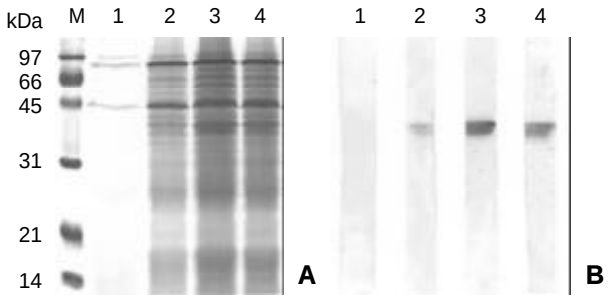

Figure 6 Induction of recombinant HMC-CP expressed in *P. pastoris*. A: secreted proteins analyzed by SDS-PAGE. B: Western blot analysis of secreted proteins with HMC-specific monoclonal antibody, clone CA5. M: molecular weight markers; lane 1: 0 h; lane 2: 24 h; lane 3: 48 h; lane 4: 72 h.

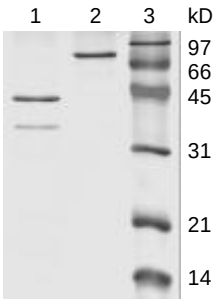

Figure 7 SDS-PAGE analysis of fusion protein cleavage (lane 1: cleaved by factor Xa; lane 2: uncleaved by factor Xa; lane 3: molecular weight marker).

Purification of rhMC-CP

Maltose-binding protein (MBP) was used as a fusion partner to provide a "tag" which could be used for the subsequent purification. The yield of the recombinant fusion protein was 12 mg/L of bacterial culture. The purified fusion protein showed a single protein band of approximately 80 kDa on SDS-PAGE.

After the fusion protein cleavage, SDS-PAGE analysis showed that the fusion protein was completely cleaved by factor Xa (Figure 7). The cleavage mixtures were loaded to heparin agarose, and the target protein showed one band about 36 kDa on SDS-PAGE, which was corresponding to the molecular weight of the native hMC-CP published previously (Figure 8A). About 1.2 mg pure recombinant protein was obtained from 5 mg fusion protein following the above procedures. The Western blot showed that this 36 kDa protein band strongly

reacted to CA5 (Figure 8B), suggesting that the recombinant protein had a good immunology activity. The N-terminal sequence of the purified recombinant protein expressed in *E.coli* was IPGRHSYAKY, and no additional amino acids were found at the N-terminus.

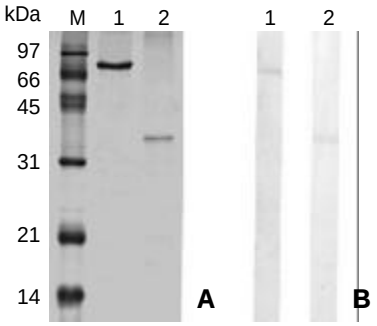

Figure 8 Analysis of purified recombinant protein. A: SDS-PAGE analysis of purified recombinant protein. B: Western blot analysis of purified fusion protein with CA5. Lane 1: purified fusion protein after MBP affinity chromatography; lane 2: purified recombinant hMC-CP after heparin agarose affinity chromatography.

Analysis of enzymatic activity

The purified recombinant hMC-CP expressed in *E.coli* had a very low level of enzymatic activity. In contrast, enzymatic activity in cell-free supernatant of *P. pastoris* culture was 11.7 U/mg secreted protein.

DISCUSSION

A cDNA of human colon hMC-CP was cloned and active enzyme was expressed in the current study, which will offer an essential tool for investigating the functions of hMC-CP, a zinc containing metalloexopeptidase.

Our result revealed that the human colon MC-CP cDNA comprised 1 251 bp, which agreed with the skin and lung mast cell carboxypeptidase[22,23,26]. The hMC-CP was predicted to be translated as a 417 amio acid preproenzyme which includes a 15 amino acid signal peptide, a 94 amino acid activation peptide and 308 amino acid mature mast cell carboxypeptidase. When comparison of the DNA sequence of human colon MC-CP cDNA with skin MC-CP cDNA, five variations were found which caused 3 amino acid substitutions, but there was Not any difference between the human colon and skin MC-CP. The meaning of these variations between tissues in man requires more investigations.

Since the role of hMC-CP in man remains unclear and human mast cells contain large amount of MC-CP, there is a pressing need to investigate the functions of this enzyme. One of the difficulties in investigating the potential functions of MC-CP over the years was that it was uneasy to obtain a substantial quantity of the active enzyme. Purification of MC-CP from human tissues was not only hard to perform, but also difficult to collect enough tissues for purification. Therefore, development of an efficient heterologous expression system for the production of recombinant hMC-CP is an alternative for obtaining a sufficient quantity of hMC-CP. There are a number of options for heterologous recombinant expressions, among them *E.coli* expression system is the most convenient and frequently used, therefore, *E.coli* expression system was used to express hMC-CP. The pMAL-C2x plasmid[36,37], a vector that allows the fusion of the target protein N-terminus to the MBP tag, made the purification of recombinant proteins much easier.

The extra residue(s) is often added to the C-terminus or N-terminus of recombinant protein. In this study, a pair of specific primers were designed. The upstream primer contained the sequences for a factor Xa recognition site just before the sequence for N-terminus of hMC-CP, and the downstream primer contained a terminator. The PCR product was inserted into the expression vector pMAL-c2x, which yielded the recombinant protein without extra residues after the fusion protein was cut with factor Xa. The result of N-terminal amino acid sequencing also showed that the N-terminus of recombinant hMC-CP had no extra residue.

After induction with IPTG, the recombinant protein was expressed in *E.coli*, with a molecular weight of about 80 kDa. This was in agreement with the expected molecular mass of the fusion proteins MBP (45 kD) and HMC-CP (36 kD). It was reported that the target gene fused to bacterial gene could improve the expression level and increase the solubility of recombinant proteins[37]. The expression vector pMAL-2x containing *malE* gene of *E.coli* encoding MBP was used for fusion expression. The target gene was inserted downstream from the *malE* gene, which resulted in the expression of hMC-CP fused to MBP. The solubility of recombinant proteins generally could be increased when the cell culture temperature decreased[38]. In our case, although the culture temperature was reduced to 23 ℃, insoluble recombinant proteins were still the major products. Since purification of recombinant proteins from inclusion body was a complicated process, we only used soluble products to isolate active recombinant hMC-CP.

Fusion protein was purified with one–step affinity chromatography with maltose. Once the fusion protein was isolated, it was necessary to remove the tag. In this study, the linker sequence recognized by factor Xa was designed between the MBP and target protein, because there were no such sequences in MBP and hMC-CP. After the fusion protein cleavage, usually ion exchange chromatography and hydroxyapatite chromatography were used in separating the protein of interest from MBP[36,39]. But in this study, the recombinant protein was purified by heparin agarose chromatography as MC-MBP could tightly bind to heparin[40]. In comparison with ion exchange chromatography and hydroxyapatite chromatography, heparin agarose chromatography was simpler and more convenient. Approximately1.2 mg target protein was obtained from 5 mg fusion protein following the established procedures. N-terminal amino acid sequencing showed that the first 10 amino acids of the recombinant hMC-CP were in good agreement with the human skin and lung MC-CP. Western blotting analysis showed that the recombinant protein had the similar immuno-reactivity with its natural counterpart, indicating that the recombinant hMC-CP could be used as an antigen to produce a specific antibody.

Our studies revealed that the purified recombinant hMC-CP expressed in *E.coli* had a very low level of enzymatic activity to substrate hippuryl-L-phenylalanine. It might be possible that the *E. coli* expression system is a prokaryotic expression system, which can not carry out post-translation modifications. In order to obtain higher levels of enzymatic activity of recombinant hMC-CP, we used *P. pastoris* to express hMC-CP. The enzymatic assay showed that the hMC-CP expressed in *P.asptoris* had a relatively high activity (11.7 U/mg secreted protein) towards hippuryl-L-phenylalanine. It is possible that *P.asptoris* is an eukaryotic expression system, which has the ability to perform eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing[41]. Our result showed that the supernatant of *P.pastoris* culture had the highest enzymatic activity on the second day after induction by methanol, the enzymatic activity would decrease when induction time increased. It is possible that the secreted recombinant protein was degraded with the increase of induction time.

In conclusion, cDNA encoding human colon MC-CP can

be cloned and expressed in *E.coli* and *P.asptoris*. The expression of recombinant hMC-CP can facilitate its functional study including its role in intestinal diseases.

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