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Purification and molecular cloning of chymase from human tonsils

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A chymotrypsin-like protease was purified to homogeneity from human tonsils by a series of chromatographic procedures. The purified enzyme gave a single protein band with an apparent molecular mass of 30 kDa on SDS-PAGE. The sequence of the first 21 amino acids at the N-terminus of the enzyme was determined. A cDNA for the enzyme was cloned by PCR amplification from extracted tonsillar mRNA using a supposed N-terminal oligonucleotide primer and a conserved C-terminal primer of the chymase family. The deduced amino acid sequence of the isolated clone was identical to that of human chymase in connective tissue-type mast cells from heart except for a Ser instead of a Cys at the N-terminal 7th position.

Chymase; Human tonsil, cDNA cloning; Amino acid sequence

1. INTRODUCTION

Human mast cells play a crucial role in allergic inflammation. A chymotrypsin-like protease, chymase, in the histamine granules of mast cells has been postulated to play a role in the stimulation–secretion coupling or the exocytotic process of histamine granules induced by IgE [1–3]. Immunohistochemical studies have demonstrated the presence of two types of mast cells, (i) connective tissue mast cells with chymase in the skin and submucosa of the gut and (ii) mucosal mast cells lacking chymase in the lung and gut mucosa [4]. Human chymase has been purified from the heart [5] and skin [6] and cDNA for human heart chymase has been cloned [7], although dispersed mast cells from these human organs have not been prepared.

In order to study the mechanism of degranulation from human mast cells and to identify the inhibitors of degranulation as anti-allergic drugs, human mast cells prepared from operatively removed tonsils and adenoids are particularly useful [3]. At present, however, little is known about the chymotrypsin-like protease in human tonsillar mast cells. We therefore purified this enzyme from human tonsils and cloned the cDNA by PCR amplification. In this paper, we are the first to describe the amino acid sequence of the chymotrypsin-like protease from human tonsils, which is classified into the family of human chymases.

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Abbreviations: HPLC, high performance liquid chromatography; Suc, Succinyl, MCA, 4-methylcoumaryl-7-amide; PCR, polymerase chain reaction; bp, base pair; kDa, kilodaltons; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

2. MATERIALS AND METHODS

2.1 *Materials*

Surgically removed tonsils from children (3–4 g/piece) were studied. G2000SW-XL and a Heparin 5PW HPLC column were obtained from Tosoh Inc., Tokyo, Japan. The Aquapore RP-300 7C8 reverse-phase HPLC column was from Applied Biosystems Inc., USA. Suc-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA) was from Protein Research Foundation, Osaka, Japan. Membranes were from Amicon Inc., USA. The primers were synthesized on an Applied Biosystems 391 PCR-mate. Isolated DNAs were sequenced using an Applied Biosystems model 370A DNA Sequencer. All other materials were of commercial grade.

2.2. *Enzyme and protein assays*

The activity of enzymes was measured with Suc-LLVY-MCA in 0.1 M phosphate buffer, pH 8.0 [8]. The hydrolyzed product, 7-amino-4-methyl-coumarin, was detected by fluorescence spectrophotometry (Hitachi Inc., Japan) with emission at 460 nm and exitation at 370 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmol of the substrate per min. The protein concentration was determined by the Lowry method [9].

2.3. *Enzyme purification*

The following steps were all performed at 4°C. The human tonsils (60 g) were minced and homogenized with 600 ml of 0.1 M phosphate buffer, pH 8.0, in a Polytron for 5 min, and the homogenates were centrifuged at 22,000 × *g* for 30 min. This procedure was repeated twice to remove soluble cellular proteins. A chymotrypsin-like protease that hydrolyzed Suc-LLVY-MCA was extracted by homogenization with 600 ml of 0.1 M phosphate buffer, pH 8.0, containing 2 M NaCl and 5% ammonium sulfate. The extract was centrifuged at

27,000 × *g* for 20 min and the supernatant was ultrafiltrated through an Amicon YM100 membrane. The filtrate was concentrated on an Amicon YM10 and subjected to gel-permeation HPLC on a G2000SW-XL column (6.0 × 300 mm), pre-equilibrated with the enzyme extraction buffer. The enzyme was eluted with an apparent molecular mass of about 30 kDa. The active fractions were pooled and concentrated by Amicon YM10. The concentrated samples was diluted 20 times with 25 mM Tris-HCl buffer, pH 8.5, to adjust the NaCl concentration at 0.1 M. The diluted fraction was applied to a Heparin 5PW HPLC column (7.5 × 75 mm) and the column was washed with 25 mM Tris-HCl, pH 8.0, 0.1 M NaCl until the absorbance of the eluate at 280 nm had returned to the baseline. Then the enzyme was eluted with a linear gradient of 0.1–2.0 M NaCl in 25 mM Tris-HCl buffer, pH 8.0. The active fractions that eluted at about 1.2 M NaCl were pooled, concentrated and desalted with 25 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, on the Amicon YM10. At this step, the enzyme migrated as a single protein band with an apparent molecular mass of 30 kDa on SDS-PAGE under reducing conditions followed by staining with Coomassie brilliant blue R-250 (data not shown). An aliquot of the final preparation was applied to an Aquapore RP-300 reverse-phase HPLC column to test the purity and to remove a minor contaminant. The column was equilibrated with 0.05% TFA and the enzyme was eluted with a 50 min linear gradient of 0.05% TFA–0.075% TFA containing 70% acetonitrile at a flow rate of 0.4 ml/min and 50°C. The active fraction eluted at 42 min, was concentrated on the Amicon YM 10 and the N-terminus was sequenced on an Applied Biosystems model 470 gas-phase protein sequencer

2.4. *Molecular cloning*

The enzyme gene was cloned by PCR [10]. The 5′ primer was selected from the deduced nucleotide sequence of the N-terminal 8 amino acid residues. The 3′ primer was designed to match the conserved region in the human chymase family [7]. The mRNA was prepared from fresh tonsils with guanidin isothiocyanate [11]. The mRNA was first transcribed to DNA using reverse transcriptase and an oligo-dT primer, then the resulting single-stranded cDNA was amplified using the sense strand, ATCATCGGGGGCACAGAATCCAAG, and the antisense-strand, TAATTTGCCTGCAGGATCTGGTT. A 681 bp product was obtained, subcloned into M13mp10 at the *Hin*cII site, then sequenced [12].

3. RESULTS AND DISCUSSION

3.1. *Purification of a chymotrypisin-like protease from human tonsils*

The results of a typical enzyme purification are summarized in Table I. Starting from 60 g of human tonsils, 33 μg of purified enzyme was obtained at 2.2% yield in activity with a 101,000-fold purification from the crude extract filtered through Amicon YM 100. A chymotrypsin-like protease in human tonsils was extracted with a high concentration of salt (2.0 M NaCl and 5% ammonium sulfate) after washing the homogenate with 100 mM potassium phosphate buffer, pH 8.0. This washing step should remove other neutral serine proteases, but not the enzyme. The enzyme had high affinity for heparin, eluting at 1.2 M NaCl from a heparin-Sepharose column, resulting in a 404-fold increase in specific activity. The affinity of the enzyme for heparin and the elution profile from the heparin-Sepharose column are identical to those of chymase from human heart [5]. The purified enzyme has an apparent molecular mass of 30 kDa on SDS-PAGE under reducing conditions, which is also identical to that of human chymase from skin and heart [5,6].

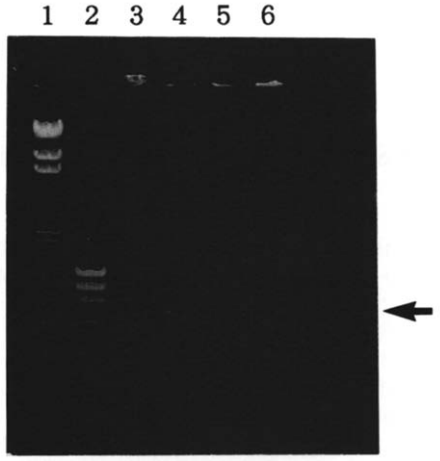

Fig. 1. Agarose gel electrophoresis of PCR products from tonsillar mRNA. Tonsillar mRNA was reverse transcribed using reverse transcriptase XL (Life Science Co., USA). The PCR reaction proceeded for 30 cycles under conditions of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extention at 72°C for 3 min, using the 5′ primers ATC ATC GGG GGC ACA GAA TCC AAG (DNA1) and ATC ATC GGG GGC ACA GAA TGC AAG (DNA2), and the 3′ primers TTA ATT TGC CTG CAG GAT CTG GTT (DNA3) and TA ATG GGA GAT TCG GGT GAA GAC (DNA4). Lane 1, λDNA-*Hin*dIII fragments; lane 2, ΦX174DNA-*Hae*III fragments; lane 3, products of DNA1 and 3; lane 4, DNA1 and 4; lane 5, DNA2 and 3; lane 6, DNA2 and 4; on 0.8% agarose.

3.2. *N-Terminal sequence*

The sequence of the 21 amino acid residues at the N-terminus of the purified enzyme was determined and

Table I

Purification of chymotrypsin-like protease from human tonsils

Purification step	Total volume (ml)	Total protein (mg)	Activity (mU)[a]	Specific activity (mU/mg)[a]	Recovery (%)
1. Amicon YM100	600	600	150,000	250	100
2. Amicon YM10	4.5	22.5	23,900	1,060	16
3. G2000 SW-XL	1.6	1.5	5,980	3,990	4.0
4. Heparin 5PW	0.2	0.033	3,340	101,000	2.2

[a]Units of Suc-LLVY-MCA hydrolyzing activity

Table II

N-Terminal sequences of human tonsillar chymotrypsin-like protease and chymases from various sources

1. Human tonsillar enzyme	IIGGTESKPH SRPYMAYLEI V
2. Human skin chymase[a]	IIGGTESKPH SRPYMAYLEI V
3. Human heart chymase[b]	IIGGTECKPH SRPYMAYLEI V
4. Rat chymase[c]	IIGGVESRPH SRPYMAHLEI T
5. Mouse chymase[d]	IIGGVEARPH SRPYMAHLEI T
6. Dog chymase[e]	IIGGTKSKPH SRPYMAHLEI L

Refer to [a][6], [b][7], [c][12], [d][13], and [e][14].

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        10         20         30         40         50         60
ATCATCGGGGGCACAGAATCCAAGCCACATTCCCGCCCCTACATGGCCTACCTGGAAATT
I  I  G  G  T  E  S  K  P  H  S  R  P  Y  M  A  Y  L  E  I

        70         80         90        100        110        120
GTAACTTCCAACGGTCCCTCAAAATTTTGTGGTGGTTTCCTTATAAGACGGAACTTTGTG
V  T  S  N  G  P  S  K  F  C  G  G  F  L  I  R  R  N  F  V

       130        140        150        160        170        180
CTGACGGCTGCTCATTGTGCAGGAAGGTCTATAACAGTCACCCTTGGAGCCCATAACATA
L  T  A  A  H  C  A  G  R  S  I  T  V  T  L  G  A  H  N  I

       190        200        210        220        230        240
ACAGAGGAAGAAGACACATGGCAGAAGCTTGAGGTTATAAAGCAATTCCGTCATCCAAAA
T  E  E  E  D  T  W  Q  K  L  E  V  I  K  Q  F  R  H  P  K

       250        260        270        280        290        300
TATAACACTTCTACTCTTCACCACGATATCATGTTACTAAAGTTGAAGGAGAAAGCCAGC
Y  N  T  S  T  L  H  H  D  I  M  L  L  K  L  K  E  K  A  S

       310        320        330        340        350        360
CTGACCCTGGCTGTGGGGACACTCCCCTTCCCATCACAATTCAACTTTGTCCCACCTGGG
L  T  L  A  V  G  T  L  P  F  P  S  Q  F  N  F  V  P  P  G

       370        380        390        400        410        420
AGAATGTGCCGGGTGGCTGGCTGGGGAAGAACAGGTGTGTTGAAGCCGGGCTCAGACACT
R  M  C  R  V  A  G  W  G  R  T  G  V  L  K  P  G  S  D  T

       430        440        450        460        470        480
CTGCAAGAGGTGAAGCTGAGACTCATGGATCCCCAGGCCTGCAGCCACTTCAGAGACTTT
L  Q  E  V  K  L  R  L  M  D  P  Q  A  C  S  H  F  R  D  F

       490        500        510        520        530        540
GACCACAATCTTCAGCTGTGTGTGGGCAATCCCAGGAAGACAAAATCTGCATTTAAGGGA
D  H  N  L  Q  L  C  V  G  N  P  R  K  T  K  S  A  F  K  G

       550        560        570        580        590        600
GACTCTGGGGGCCCTCTTCTGTGTGCTGGGGTGGCCCAGGGCATCGTATCCTATGGACGG
D  S  G  G  P  L  L  C  A  G  V  A  Q  G  I  V  S  Y  G  R

       610        620        630        640        650        660
TCGGATGCAAAGCCCCCTGCTGTCTTCACCCGAATCTCCCATTACCGGCCCTGGATCAAC
S  D  A  K  P  P  A  V  F  T  R  I  S  H  Y  R  P  W  I  N

       670        680
CAGATCCTGCAGGCAAATTAA
Q  I  L  Q  A  N  *
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Fig. 2. DNA sequence and deduced amino acid sequence of human tonsillar chymotrypsin-like protease. The 681 base cDNA was generated using primers DNA1 and DNA3 as shown in Fig. 1, lane 3. DNAs 1, 3 and 4 represented in the legend for Fig. 1 are underlined. The asterisk indicates the termination codon.

is shown in Table II, along with those of human skin and heart, as well as rat, mouse, and dog chymases. The amino acid sequence of human tonsillar chymotrypsin-like protease was identical to that of chymase from human skin [6], and that of chymase from human heart except for having Ser instead of Cys at the N-terminal 7th position [5]. It was also similar to those of chymases from rat [12], mouse [13], and dog [14].

3.3. *Molecular cloning of chymase*

Fresh human tonsils were the source of mRNA. The extracted tonsils were immediately soaked in liquid nitrogen, then mRNA was extracted in guanidine isothiocyanate [11]. The total RNA was further purified to poly(A) RNA by oligo-dT column chromatography. The mRNA (2.3 μg) was transcribed to cDNA by reverse transcriptase, then the cDNA products were used for PCR. The PCR primers were selected as follows. The 5′ primer was ATCATCGGGGGCACAGAATCCA-AG (DNA1) from the N-terminal sequence IIGGTESK. The other 5′-primer was ATCATCGGGGGCACAGAATGCAAG (DNA2) based on the N-terminal sequence of human heart chymase, in which the first 7th amino acid is Cys instead of Ser. The 3′ primers were TAATGGGAGATTCGGGTGAAGAC (DNA4) for the VFTRISHY sequence of one of

the most conserved regions of the chymase family, and TTAATTTGCCTGCAGATCTGGTT (DNA3) for the C-terminal sequence of human heart chymase [7]. A cDNA encoding the human tonsillar chymotrypsin-like enzyme was obtained by PCR using DNA1 as the 5′ primer and DNA3 or 4 as the 3′ primer, but no PCR product was amplified using DNA2 instead of DNA1 as the 5′ primer as shown in Fig. 1. The sequences of the PCR products amplified using a combination of DNA1 and 3 and that of DNA 1 and 4 are the same. The length of the PCR product amplified using DNA1 and 3 as primers was about 680 bp on 0.8% agarose. The product on the gel was excised, purified by resin, and the cDNA was blunt-ended and inserted in M13 mp10 at an *Hin*cII site. The deduced amino acid sequence of the cloned cDNA comprises 214 amino acid residues, as shown in Fig. 2. The complete amino acid sequence determined was identical to that of chymase from human heart mast cells [7] except for the substitution of Ser instead of Cys at the N-terminal 7th position, and the N-terminal 35 residues of the enzyme were identical to those of human skin chymase previously reported [6]. The results suggest that a chymotrypsin-like protease isolated from human tonsils is a member of the human chymase family found in connective tissue mast cells. The results also support the finding that mast cells prepared from human tonsils are a useful source of human chymase and for studing the inhibition of histamine release from human mast cells by anti-allergic drugs, including chymase inhibitors [1–3].

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