Previously we isolated a trypsin-like enzyme designated human airway trypsin-like protease from the sputum of patients with chronic airway diseases. This paper describes the cDNA cloning, characterization of the primary protein structure deduced from the cDNA, and gene expression of this enzyme in various human tissues. We obtained an entire 1517-base pair sequence of cDNA with an open reading frame encoding a polypeptide with 418-amino acid residues. The polypeptide consisted of a 232-residue catalytic region and a 186-residue noncatalytic region with a hydrophobic putative transmembrane domain near the NH2 terminus. The polypeptide was suggested to be a type II integral membrane protein in which the COOH-terminal catalytic region is extracellular. Therefore, this protein is thought to be synthesized as a membrane-bound precursor and to mature to a soluble and active protease by limited proteolysis. It showed 29–38% identity in the sequence of the catalytic region with human hepsin, enteropeptidase, acrosin, and mast cell tryptase. The noncatalytic region had little similarity to other known proteins. In Northern blot analysis a transcript of 1.9 kilobases was detectable most prominently in the trachea among 17 human tissues examined.

Many previous investigations have indicated that proteases released from immunoinflammatory cells participate in pathogenesis of several kinds of respiratory diseases. For instance, neutrophil elastase has been shown to be intimately related to the pathologic states of pulmonary emphysema (1, 2), cystic fibrosis (3, 4), interstitial pneumonia (5), and adult respiratory distress syndrome (6) through destruction of extracellular matrix components, such as elastin, of alveolar and bronchial tissues. Mast cells, which abound in airway mucosa and in alveolar wall, release trypsin-like protease (trypsinase) and chymotrypsin-like protease (chymase) into extracellular spaces during degranulation (7). The trypsin has potential to stimulate smooth muscle, fibroblast, and tissue turnover (8). Different substrates for chymase (9–11) indicate the potential involvement of the enzyme in a variety of processes related to the inflammatory response. Recently it was revealed that chymase from human mast cells selectively converted big endothelins to trachea-constricting peptides (12). These effects of the two mast cell proteases have attracted considerable attention as one of the pathogenic determinants and the therapeutic targets of bronchial asthma and allergic inflammation. Elastase released from alveolar macrophages has also been suggested to contribute to the pathogenesis of pulmonary emphysema by degrading matrix components of alveolar walls (13, 14).

However, there are very few reports dealing with the functions and roles of proteases secreted from respiratory tissues, such as secretary glands or surface epithelial cells of the airway. Kido and co-workers (15, 16) found a novel trypsin-like protease that is secreted from rat Clara cells, secretory cells localized to the distal airway only. The protease, named tryptase Clara, was shown to enhance the infectivity of influenza and Sendai viruses (17), although its physiological role is unknown.

Previously, we found trypsin-like activity in the sputum of patients with chronic airway diseases and isolated a novel trypsin-like protease from the sputum, designated human airway trypsin-like protease (HAT)1 (18). Gel filtration studies showed that HAT was a monomeric enzyme with an apparent molecular mass of 27 kDa. Immunohistochemical studies showed that HAT was localized mainly in cells of submucosal serous glands of the bronchi and trachea. These results indicate that HAT is released from the submucosal serous glands onto mucous membrane, at least in patients with chronic airway diseases.

In this paper, we report the cloning of HAT cDNA, the primary structure of this enzyme and characterization of the polypeptide deduced from the nucleotide sequence of the cDNA, and results of analysis of expression of HAT mRNA in various human tissues. The primary structure of HAT was compared with that of other known serine proteases.

**EXPERIMENTAL PROCEDURES**

_Materials—_Human trachea QUICK-CloneTM cDNA, human trachea poly(A)+ RNA, human trachea 11g10 cDNA library (oligosdT) and random-primed 5-RACE kit, human multiple tissue Northern blots, and human β-actin cDNA were purchased from CLONTECH Laboratories Inc. (Palo Alto, CA). Taq DNA polymerase was from Promega Corp. (Madison, WI). SureCloneTM ligation kit, dNTP, and plasmid vector pUC18 were from Amersham Pharmacia Biotech. Avian myeloblastosis virus reverse transcriptase and RNase inhibitor were from Boehringer Mannheim. Restriction endonucleases, random primer labeling kit, and Escherichia coli JM109 were from Takara Shuzo Co. Ltd. (Osaka, Japan). Nylon membrane HybondTM-N+ for blotting and [α-32P]dCTP for probe labeling in hybridization were from Amersham. Denhardt's solution and salmon sperm DNA were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Qiagen lambda kit for purification of phage DNA was used.

1 The abbreviations used are: HAT, human airway trypsin-like protease; FCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; bp, base pair(s); kb, kilobase or kilobase pair.
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from Qiagen GmbH. (Hilden, Germany). Oligonucleotide purification cartridge column and DyeDeoxy™ terminator cycle sequencing kit for sequencing of DNA were from Applied Biosystems Inc. (Foster City, CA).

DNA Amplification by Polymerase Chain Reaction (PCR)—PCR was performed according to the procedure described by Sambrook et al. (19). Oligonucleotides used as PCR primers were synthesized by a DNA/RNA synthesizer (Applied Biosystems Inc., model 394) and purified by oligonucleotide purification cartridge column. Unless otherwise stated, PCR was carried out by adding 15 pmol of each primer and an appropriate amount of template DNA to 20 μl of PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 100 μM of each dNTP, 0.1% Triton X-100, 1 U Tag DNA polymerase and 0.2 mM dNTP). The reaction using a DNA thermal cycler (Perkin-Elmer Corp.) was carried out for 35 cycles of 1-min denaturation at 94 °C, 1.5-min annealing at 57 °C, and 2-min extension at 72 °C.

Subcloning of DNA Fragments—To clone DNA fragments that were amplified by PCR, SureClone™ ligation kit was used. DNA fragments were blunted by Klenow fragment, inserted into the Smal site of plasmid vector pUC18, and introduced into E. coli JM109 by Hanahan’s method (20). On the other hand, for subcloning of insert DNA of Agt10 plage clone, the insert DNA was excised by EcoRI from phage DNA, which was purified using Qiagen lambda kit and inserted into the EcoRI site of plasmid vector pUC18. E. coli JM109 was transformed as described above. The purified DNA was isolated and transformed by the alkaline lysis procedure (21) with minor modifications.

Analysis of DNA and Amino Acid Sequence—The nucleotide sequence of the DNA inserted into plasmid vector pUC18 was analyzed by an automated DNA sequencer (Applied Biosystems Inc., model 373) using the DyeDeoxy™ terminator cycle sequencing kit. Both strands of all clones were completely sequenced. Hydropathy of amino acid sequence was analyzed (22) with the Genetyx program package (Software Development Co. Ltd., Tokyo, Japan). A computer survey of the National Biomedical Research Foundation (Washington, D.C.) and SWISS-PILOT (European Bioinformatics Institute, Geneva, Switzerland) data banks for similarity of amino acid sequences between HAT and other known proteins was carried out using MParch program, which was modified from the method of Smith and Waterman (23) with TEjin Systems Technology Ltd. (Yokohama, Japan).

Amplification of a Partial cDNA Fragment—In a previous report (18), we showed that the sequence of the 20 NH2-terminal amino acids of native HAT purified from the sputum of patients with chronic airway diseases was ILGTTGEAEESGWPGVQSLRL (amino acids 187–206 in Fig. 1). Based on this amino acid sequence, we designed and synthesized the 5′-end and 3′-end oligonucleotide primers from the upstream side of the cDNA coding for native HAT, 5′-RACE was performed and amplified DNA was isolated from each of in the reaction mixture. Using T4 RNA ligase, AmpliFINDER™ anchor was ligated to the 3′-ends of the single-stranded cDNAs. PCR amplification (0.75 min at 94 °C, 0.75 min at 57 °C, and 2 min at 72 °C) was then carried out using 0.1 pmol of each primer and an appropriate amount of template DNA to 20 μl of PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 1% Triton X-100) containing 0.5 units of DNA polymerase and 0.2 mM dNTP. The reaction using a DNA thermal cycler (Perkin-Elmer Corp.) was carried out for 35 cycles of 1-min denaturation at 94 °C, 1.5-min annealing at 57 °C, and 2-min extension at 72 °C.

Amplification of cDNA by 5′-RACE—To obtain a cDNA that had a nucleotide sequence in the upstream side of the cDNA coding for native HAT, amplification of the cDNA was carried out using 5′-RACE kit (24). Single-stranded cDNAs were synthesized by reverse transcription of 2 μg of human trachea poly(A)+ RNA using the antisense primer 5′-ACGGTGCAAATCCGTAGGGAT-3′ (nucleotides 785–761 in Fig. 1). The single-stranded cDNAs were then purified using glass powder in 50% ethanol. The purified DNA was then loaded. Amplification of cDNA by 5′-RACE was then subcloned and sequenced.

Expression and Purification of Recombinant HAT—A 1.3-kb BamHI-HindIII fragment containing the entire human HAT cDNA was cloned into a vector plasmid pBlueBacII (Invitrogen, San Diego, CA) to generate pBlueHAT1. Recombinant HAT-expressing viruses were generated after transfection of PEGF/SF9 cells with pBlueHAT1 DNA essentially as described by the manufacturer (Invitrogen). For baculovirus/insect cell expression (26), 800 ml of Tn5 (27) cells were then infected with the high titer lysate for 72 h and harvested by centrifugation. The cell pellet was treated with 1% Triton X-100 for 1 h on ice and was centrifuged at 100,000 × g for 1 h at 4 °C. From this infected cell lysate, the recombinant HAT was isolated by sequential chromatography, including the native HAT as described previously (18). SDS-polyacrylamide gel electrophoresis, immunoblotting, and degradation of fibrinogen by HAT were done as described (18).

Northern Blot Analysis—The expression level of HAT mRNA in various human tissues was examined by Northern blot analysis. To prepare the probe for the analysis, the full-length cDNA for HAT was 5′-labeled by random priming (25) and hybridized as follows. Northern blots, which were prepared from various tissues, which contained 2 μg of poly(A)+ RNA derived from various tissues in each lane, were probed under the same conditions as the library screening described above (except that the concentration of SDS was 0.5%) and then washed. In the case of the blot for trachea, 2 μg of human trachea poly(A)+ RNA was resolved by 1% agarose-Formaldehyde gel electrophoresis (28), and transferred onto Hybond™-N+ blotting membrane and UV-cross-linked. X-ray films were exposed to the probed blots for 4 days at –80 °C with an intensifying screen, and the presence of HAT mRNA in each human tissue was evaluated. These blots were then stripped of the HAT cDNA probe by boiling in 0.5% SDS for 10 min and re-probed with 32P-labeled human β-actin control probe as an internal standard for the amounts of RNA loaded.

RESULTS AND DISCUSSION

Cloning of HAT cDNA—Using a pair of highly degenerate oligonucleotide primers, the partial 59-bp DNA fragment for HAT, which contained a nucleotide sequence coding for the NH2-terminal 19-residue amino acid sequence of native HAT, was obtained by PCR amplification from human trachea cDNA. To stretch this cDNA sequence to the 3′-end, a 3′-RACE reaction was carried out. The resulting 0.9-kb amplified product was shown to encompass the entire nucleotide sequence of the 3′ region, including the poly(A) tail of HAT cDNA (nucleotides 635–1517 in Fig. 1). The amino acid sequence deduced

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2 Y represents T or C; N represents C or I (inosine); R represents G or A; K, G or T; M, A or C; S, G or C.

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from this 0.9-kb fragment was shown to exactly contain the 15-amino acid sequence (amino acids 192–206 in Fig. 1) of the NH2-terminal 20-amino acid sequence of the native HAT. With this 0.9-kb cDNA fragment as a probe, $1 \times 10^6$ clones of a human trachea cDNA library were screened. Five of 28 independent positive clones were then subcloned and sequenced. The largest insert was shown to contain a 1323-bp sequence of cDNA (nucleotides 133–1455 in Fig. 1) but was considered not to contain the entire nucleotide sequence of the 5'-9' region of HAT cDNA. To obtain the missing sequences in the 5'-9' region of HAT cDNA, 5'-RACE reaction was carried out. The 5'-RACE procedure produced a 741-bp cDNA fragment (nucleotides 1–741 in Fig. 1). This product had a 609-bp nucleotide sequence overlapping (nucleotides 133–741 in Fig. 1) with the 5'-end of the largest insert of cDNA clone obtained by the cDNA library screening.

**Fig. 1.** Nucleotide sequence of HAT cDNA and its deduced amino acid sequence. The nucleotide sequence of the HAT cDNA is shown along with the deduced amino acid sequence beginning with the first ATG codon. A stop codon (TAG) at the terminus of the translation sequence is marked with an asterisk. Nucleotides are numbered at the right margin and amino acids on the left. The NH2-terminal sequence obtained from the purified enzyme is underlined. The boxed amino acid sequence represents a potential transmembrane domain.

from this 0.9-kb fragment was shown to exactly contain the 15-amino acid sequence (amino acids 192–206 in Fig. 1) of the NH2-terminal 20-amino acid sequence of the native HAT. With this 0.9-kb cDNA fragment as a probe, $1 \times 10^6$ clones of a human trachea cDNA library were screened. Five of 28 independent positive clones were then subcloned and sequenced. The largest insert was shown to contain a 1323-bp sequence of cDNA (nucleotides 133–1455 in Fig. 1) but was considered not to contain the entire nucleotide sequence of the 5'-9' region of HAT cDNA. To obtain the missing sequences in the 5'-9' region of HAT cDNA, 5'-RACE reaction was carried out. The 5'-RACE procedure produced a 741-bp cDNA fragment (nucleotides 1–741 in Fig. 1). This product had a 609-bp nucleotide sequence overlapping (nucleotides 133–741 in Fig. 1) with the 5'-end of the largest insert of cDNA clone obtained by the cDNA library screening.

**Sequence and Structural Features of HAT cDNA—Analysis of the cDNA clones obtained by the successive procedures including 3'-RACE, cDNA library screening, and 5'-RACE showed a 1517-bp nucleotide sequence up to the poly(A) region (Fig. 1), which represented the HAT cDNA sequence. This nucleotide sequence was also shown to contain one open reading frame, and the polypeptide deduced from the cDNA included the 20-residue amino acid sequence of the NH2 terminus of the native HAT (amino acids 187–206 in Fig. 1). The molecular mass of the polypeptide, including the NH2 terminus of the 20 residues to the COOH terminus deduced from the stop codon TAG (nucleotide-1316), was estimated to be 25,308 Da. This value is similar to the apparent molecular mass (27 kDa) estimated by gel filtration of the native HAT protein purified from sputum (18).

In the 5'-flanking region of this cDNA, one in-frame stop codon TAG was located at nucleotide 26. Four in-frame ATG codons were detectable between this stop codon and the region encoding the native HAT, but none of these ATG codons satisfied the criteria for a Kozak consensus sequence (29). Therefore we could not determine the translational initiation site in the cDNA from the nucleotide sequence. To determine the initiation site, we expressed recombinant HAT in a baculovirus/insect cell system using the HAT cDNA. The recombinant virus containing the HAT cDNA was isolated, and the insect cell Tn5 was infected with the virus and then cultured. The lysate obtained by 1% Triton X-100 treatment of the infected cells was analyzed by immunoblotting with a rabbit antibody against a peptide corresponding to the NH2-terminal 19-amino acid sequence of the native HAT (18) as primary antibody, and the immunoblotting indicated that the infected cells biosynthesized a protein with a molecular mass of 48 kDa as a main product (Fig. 2). The molecular mass of each polypeptide, deduced from the nucleotide sequence initiating from each of 4 ATG codons in the cDNA, was 46,263, 32,933, 31,436, and 30,107 Da, respectively. The molecular mass of 46,263 Da is the most similar to that of the recombinant protein expressed in the insect cells, suggesting that the ATG located nearest the 5'-end (at nucleotide 62) is the initiation codon of HAT.

To demonstrate that the cloned enzyme has the same activity as the native HAT, the recombinant HAT that was expressed in the baculovirus/insect cell system was isolated in its active form. The minor product in Fig. 2, lane 3 was isolated selectively as the active recombinant HAT from the infected cell lysate by sequential chromatographic procedures of the native HAT.
HAT purification (18). The purified recombinant enzyme has the molecular mass of 28 kDa on SDS-polyacrylamide gel electrophoresis and the identical 10 NH₂-terminal residues to the native HAT. Immunoblotting also showed the purified recombinant enzyme as same size as the native HAT (Fig. 2). The recombinant HAT had an enzymatic activity degrading fibrinogen, especially the α-chain (Fig. 3), similar to the native HAT. Immunoblotting also showed the purified recombinant enzyme as same size as the native HAT (Fig. 2). The recombinant HAT had an enzymatic activity degrading fibrinogen, especially the α-chain (Fig. 3), similar to the native HAT.

From these results, it was established that the isolated cDNA clone encodes HAT. Based on these results, the nucleotide sequence of the cDNA for HAT (Fig. 1) was summarized as follows. The cDNA includes 1254 nucleotides coding for 418 amino acids and two untranslated nucleotide sequences composed of 61 and 185 nucleotides at the 5’- and 3’-end, respectively. In the 3’-untranslated region, there is a polyadenylation signal sequence, ATTAAA, at nucleotides 1478–1483, 17 nucleotides distant from the poly(A) tail.

Analysis of Deduced Amino Acid Sequence of HAT—The open reading frame of HAT cDNA was thought to encode a polypeptide consisting of 418 amino acid residues, thus having the molecular mass of 46,263 Da. The NH₂-terminal 20-amino acid sequence of the native HAT extends from Ile187 to Leu206 in the sequence of the deduced polypeptide (Fig. 1). This result indicates that the Arg186–Ile187 peptide bond in the HAT polypeptide should be cleaved for activation of HAT. This type of cleavage has been shown to be a relatively common step for activation of many known serine protease zymogens (30, 31). Therefore it is likely that the HAT gene product is synthesized as a precursor protein that consists of a noncatalytic region with 186 amino acid residues (20,955 Da, amino acids 1–186 in Fig. 1) and a catalytic region with 232 amino acid residues (25,308 Da, amino acids 187–418 in Fig. 1) and that the precursor is converted to an active enzyme by limited proteolysis like trypsinogen to trypsin in the small intestine (32). In this noncatalytic region, there were two potential N-linked glycosylation sites, namely Asn-Asn-Ser and Asn-Pro-Ser, at Asn144 and Asn152, respectively.

A hydropathy plot (22) of the predicted amino acid sequence of HAT precursor (Fig. 4) showed that a typical NH₂-terminal signal sequence (33–35) is not present, but a single obvious hydrophobic region (amino acids 13–43 in Fig. 1) is present near the NH₂ terminus. This hydrophobic region consisting of 31 amino acid residues does not contain any charged amino acids and is flanked by charged amino acids (Arg12 and Asp44). This internal hydrophobic region is thought to correspond to a transmembrane domain that anchors the protein to the cell membrane (36). A generalized rule in the eucaryotic transmembrane proteins (37, 38) suggests that the difference in total charge between 15-residue sequences on either side of the membrane-spanning hydrophobic region determines the orientation of the protein, with the more positive side facing the cytosol. As for the precursor polypeptide deduced from HAT cDNA, the NH₂-terminal side of the hydrophobic region had a net charge of +3, whereas the opposite side had that of +1. The charge on the NH₂-terminal side was +2, as positive as that on the COOH-terminal side. This result suggests that HAT precursor has an intracellular NH₂-terminal tail region consisting of 12 amino acid residues facing the cytosol and an extracellular COOH-terminal region consisting of 375 amino acid residues and containing the catalytic region. Therefore, the HAT precursor can be classified as a type II integral membrane protein (39, 40) and is thought to be synthesized as a membrane-bound precursor protein translocated to the cell surface, processed to a soluble form, and released.

Because neither the precursor nor intermediate form of HAT

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Degradation of human fibrinogen by the native HAT and the recombinant HAT. Hydrolyzing reaction and SDS-polyacrylamide gel electrophoresis were done as described previously (18). For each reaction, 0.10 μg of HAT was used. Lane 1, standard proteins; lane 2, fibrinogen (blank control); lane 3, fibrinogen hydrolyzed by native HAT; lane 4, fibrinogen hydrolyzed by recombinant HAT.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Hydropathy plot of the deduced amino acid sequence of HAT. The method of Kyte and Doolittle (22) was used with averaging over a window of 10 residues. Hydrophobic residues show positive values, whereas hydrophilic residues show negative values. Amino acid numbering begins with the start codon Met.
has been isolated and characterized, it is unknown whether or not the membrane-bound HAT is active on the cell surface. The mechanisms of expression and activation of many serine proteases have been clarified. The predicted maturation process of HAT precursor described above is similar to that of the Bacillus amyloliquefaciens subtilisin (41). The subtilisin is synthesized as a membrane-associated precursor (preprosubtilisin) and released outside the cell after it is autocatalytically converted to an active form (42). Only mature subtilisin has been detected extracellularly (41). Active HAT contained in sputum samples was also detected extracellularly.

It is possible that the membrane-bound HAT or the portion remaining in the membrane after release of the soluble HAT may be involved in some important physiological processes on the cell surface through interaction with ligands, other proteins, or the surface. Recent reports have shown that some viruses and a bacterial toxin utilize cell surface proteases as receptors (43–47), indicating other usage in addition to intrinsic roles of these proteins.

Homology of Amino Acid Sequence of HAT with Other Proteases—To find any similarity in the primary structure between HAT and known proteins, we surveyed publicly available data banks. Previous investigators have shown that the serine protease family has a common catalytic site consisting of
three amino acid residues, His, Asp, and Ser, joined by hydrogen bonds to display catalytic action as a catalytic triad, although they are located apart from each other in the primary structure of the enzyme (48). Based on these established facts, the catalytic site of HAT is thought to consist of amino acid residues His227, Asp272, and Ser368 (Fig. 5). In comparison of the amino acid sequence of HAT with those of other serine proteases, the most striking similarity was found around this putative catalytic triad as shown in Fig. 5. Six of seven cysteine residues in the catalytic region of HAT were at identical positions as those of other serine proteases (Fig. 5). Nine cysteine residues were contained in the deduced polypeptide of HAT precursor, and the Cys20 was located in the predicted transmembrane domain. Based on the locations of the known disulfide bridges in other serine proteases (49), it is postulated that the other eight cysteine residues may form four disulfide bonds, which are located at cysteine pairs 212/228, 337/353, and 364/393 in the catalytic region and at 173/292 between the noncatalytic region and the catalytic region.

It was shown that the amino acid sequence of the catalytic region of HAT was homologous to that of the other human serine proteases: 38% identity with hepsin (50), 32% with enteropeptidase (51), 30% with acrosin (52), and 29% with mast cell tryptase (53). Hepsin, of which the catalytic region shows the highest similarity with that of HAT in this survey, is a cell surface protease widely expressed in various tissues including liver and is suggested to play a role in cell growth and maintenance of cell morphology (54).

On the other hand, the amino acid sequence of the noncatalytic region of HAT showed no significant similarity with those of other proteases and had neither kringle nor an EGF-like lytic region of HAT showed no significant similarity with those of other proteases and had neither kringle nor an EGF-like lytic region of HAT showed no significant similarity with those of other proteases and had neither kringle nor an EGF-like lytic region of HAT showed no significant similarity with those of other proteases and had neither kringle nor an EGF-like lytic region of HAT showed no significant similarity with those of other proteases and had neither kringle nor an EGF-like

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