Modulation of enzymatic activity of human mast cell tryptase and chymase by protease inhibitors[1]

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ABSTRACT

AIM: To investigate the actions of protease inhibitors on the enzymatic activities of tryptase and chymase in similar experimental systems. **METHODS:** Human lung tryptase and human skin chymase were purified by a similar procedure involving high salt extraction of tryptase, heparin agarose affinity chromatography, and S-200 Sephacryl gel filtration chromatography. Actions of protease inhibitors on tryptase and chymase activities were examined by enzyme assays. **RESULTS:** The specific activities of tryptase and chymase were 2.1 kU/g protein and 4.9 kU/g protein, respectively. Both preparations showed a single diffuse band on SDS-PAGE. Among non-native protease inhibitors, *N*-(1-hydroxy-2-naphthoyl)-*L*- arginyl-*L*-prolinamide hydrochloride (HNAP), leupeptin, antipain, benzamidine, and protamine inhibited more than 90 % enzymatic activity of tryptase, whereas soy bean trypsin inhibitor (SBTI), Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPPM) and chymostatin inhibited more than 95 % enzymatic activity of chymase. Native protease inhibitors α_1-antitrypsin and secretory leukocyte protease inhibitor (SLPI) inhibited more than 90 % enzymatic activity of chymase, but lactoferrin appeared to enhance chymase enzymatic activity. All the 3 inhibitors had weak inhibitory actions on tryptase. **CONCLUSION:** The protease inhibitors tested had relatively good selectivity to either tryptase or chymase.

INTRODUCTION

Tryptase is a tetrameric serine proteinase constituting some 20 % of the total protein within human mast cells and is stored almost exclusively in the secretory granules of mast cells[1] in a catalytically active form[2]. Upon degranulation, tryptase is released from mast cells along with chymase, histamine, heparin, and other mast cell granule products[3]. Relatively high concentrations of tryptase have been detected in the serum from cases of systemic anaphylaxis[4], in bronchoalveolar lavage fluid from patients with bronchial asthma[5], in nasal lavage fluid of patients with allergic rhinitis[6], and in synovial fluid from patients with arthritis[7].

This major secretory product of the human mast cell may be a key mediator of allergic inflammation and a promising target for therapeutic intervention[8] as it has been found to be able to induce microvascular leakage in the skin of guinea pig[9], bronchoconstriction[10] in allergic sheep airways, inflammatory cell accumulation in peritoneum of mouse[11] and release of IL-8 from epithelial cells[12].

Chymase is exclusively located in the same granule as tryptase and could be released from granule together with other preformed mediators. Large quantity of active form chymase (10 pg per mast cell) in mast

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cells[13] implicates that this mast cell unique mediator may play a role in mast cell-related diseases. Indeed, chymase has been found to be able to induce microvascular leakage in the skin of guinea pig[14] and inflammatory cell accumulation in mouse peritoneum[15]. It can activate IL-1β[16] and participate in the activation-secretion process of mast cells[17]. As for tryptase, chymase could also be a key mediator of allergic inflammation and a promising target for therapeutic intervention. A recent report that the anti-inflammatory action of an effective anti-asthma drug, theophylline in asthma might result from reduction of IL-5 production in the airways[18] suggested further that mast cells were key cell types in the pathogenesis of asthma, as mast cells were reported as one of the major sources of IL-5 production in man[19] and theophylline could stabilize mast cells[20].

Research on mast cell tryptase and chymase has been carried out for approximately 20 years though it has been progressing relatively slowly and little has been done in China. This might result from lack of effective methods to purify sufficient amount of high quality tryptase and chymase. Comparing the potency of known inhibitors of tryptase and chymase is a key issue for the inhibitor drug development. However, the information on this is surprisingly limited, as there are no standard enzymes and substrates being used in the studies from different laboratories. Therefore, the aim of the current study is to investigate the actions of various protease inhibitors on the enzymatic activities of tryptase and chymase under similar experimental conditions and provide more comparable information for the inhibitor drug development.

MATERIALS AND METHODS

Purification of tryptase Human lung tissues dissected from lobectomy of the patients with lung cancer and human skin tissues were collected from the Pathology Department, Medical College, Shantou University, and were minced into small pieces. Having been washed with low salt buffer containing 2-(*N*-morpholino)ethanesulphonic acid (MES) 10 mmol/L (pH 6.1) and NaCl 0.15 mol/L, the tissues were extracted with high salt buffer containing MES 10 mmol/L (pH 6.1) and NaCl 2 mol/L. The high salt extract was then applied to heparin agarose (Sigma) in an equilibration buffer containing MES 10 mmol/L (pH 6.1) and NaCl 0.4 mol/L, and eluted from heparin agarose by NaCl 0.4-1.5 mol/L gradients. The fractions containing high tryptase activity were pooled together and concentrated to 1 mL. The concentrated eluent was then applied to S-200 Sephacryl agarose (Sigma) in a buffer containing MES 100 mmol/L (pH 6.1) and NaCl 2 mol/L. Finally, the fractions containing high tryptase activity were collected and stored at -80 ° C. The procedures above were mainly performed at 4 ° C.

Tryptase activity and purity determination Tryptase activity was determined[11] by its ability to cleave a synthetic substrate *N*-benzoyl-*D,L*-arginine-*p*-nitroanilide (BAPNA, Sigma) 2 mmol/L in Tris-HCl 0.1 mol/L (pH 8.0) and glycerol 1 mol/L at 410 nm. Protein concentration was determined by Coomassie brilliant blue G method (Pierce, USA). The specific activity of tryptase was expressed as kU tryptase activity per g protein, where one unit (U) of enzyme was taken as the amount that catalyzed the cleavage of 1 μmol of BAPNA per minute at 25 ° C. The purity of tryptase was estimated with the numbers of diffuse bands on 10 % sodium dodecyl sulphate-polyacrylamine gel electrophoresis (SDS-PAGE). The identity as tryptase was confirmed by Western blotting with monoclonal antibody (AA5, a gift from Dr Andrew F WALLS, University of Southampton, UK) against human tryptase. Tryptase prepared for this study had a single diffuse band on SDS-PAGE and its specific activity was 2.1 kU/g protein.

Isolation of chymase The procedure for isolation of chymase was described previously[14]. It involves a high salt extraction, a heparin affinity chromatography, and a S-200 Sephacryl gel filtration chromatography procedure.

Chymase activity and purity determination Enzymatic activity was determined spectrophotometrically (410 nm) by the rate of hydrolysis of *N*-succinyl-*L*-Ala-*L*-Ala-*L*-Pro-*L*-Phe-*p*-nitroanilide (SAAPP, Sigma) 0.7 mmol/L in NaCl 1.5 mol/L and Tris 0.3 mol/L (pH 8.0), and protein concentration was determined by Coomassie brilliant blue G method. The specific activity of chymase was expressed as kU activity per g protein, where 1 U of enzyme represents that required to hydrolyse 1 μmol of SAAPP per minute at 25 ° C. Purity was evaluated for tryptase and the identity of the protein band was confirmed by Western blotting with monoclonal antibody (CC1, a gift from Dr Mark G BUCKLEY, University of Southampton, UK) against human chymase. Chymase prepared for this study had a single diffuse band on SDS-PAGE and its specific

activity was 4.9 kU/g protein.

Actions of protease inhibitors on tryptase and chymase activities Enzyme and inhibitor were made up to required dilutions in normal saline. When needed, enzyme and inhibitor were mixed and incubated on ice for 30 min for non-native protease inhibitors or 20, 40, and 80 min for native protease inhibitors. Assays were performed in 96-well microtitre plates by addition of 10 μL mixture of enzyme and inhibitor plus 90 μL substrate solution, which gave a total reaction volume of 100 μL per well. The absorbance at 410 nm was monitored with a ThermoMAX plate reader (Molecular Devices). All experiments were performed in triplicate.

RESULTS

The ionic gradient applied to heparin agarose was effective in separating tryptase from irrelevant proteins. Fractions 21-26 were highly purified tryptase. Approximately 3.0-fold increase in the purity of tryptase and 75 % recovery of the activity of tryptase were achieved by heparin affinity chromatography (Fig 1). A further 2.5-fold increase in the specific activity of tryptase with approximately 70 % recovery of tryptase activity was obtained by S-200 Sephacryl gel filtration chromatography. Tryptase showed only a single diffuse band with an apparent molecular weight (M_r) of approximately 32 000 (representing disassociated monomers) on 10 % SDS-PAGE (Fig 2A). The identity of tryptase was confirmed by Western blotting with an anti-tryptase monoclonal antibody AA5 (Fig 3).

The ionic strengths applied to heparin agarose were

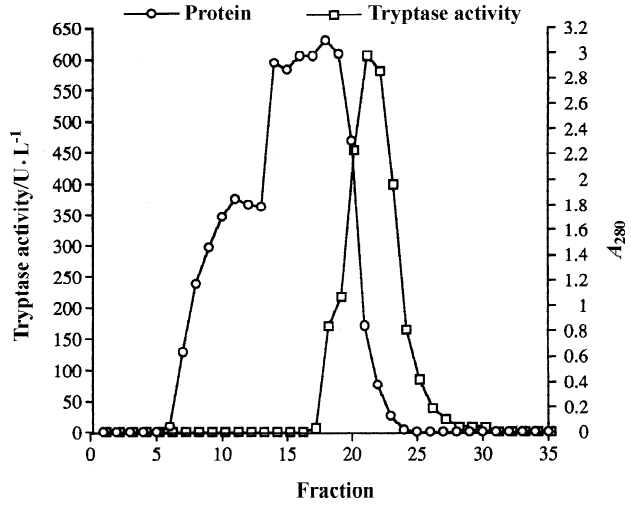

Fig 1. Isolation of tryptase from human lung tissue by heparin affinity chromatography.

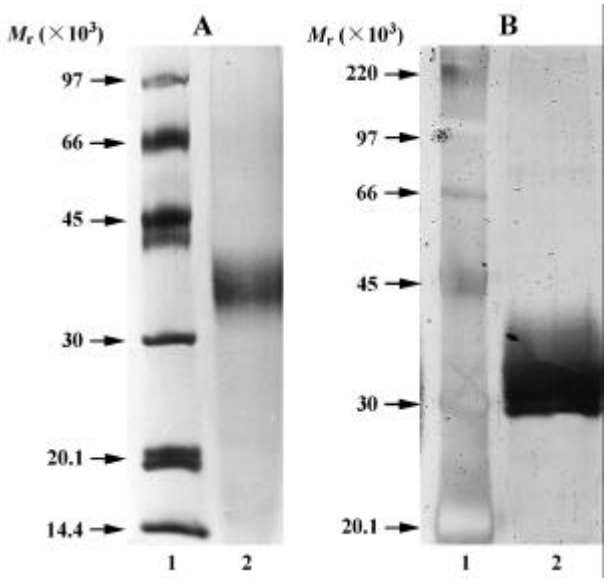

Fig 2. Appearance of (A) tryptase (monomer), Lane 1: M_r markers, Lane 2: Tryptase and (B) chymase on 10 % SDS-PAGE, Lane 1: M_r markers, Lane 2: Chymase.

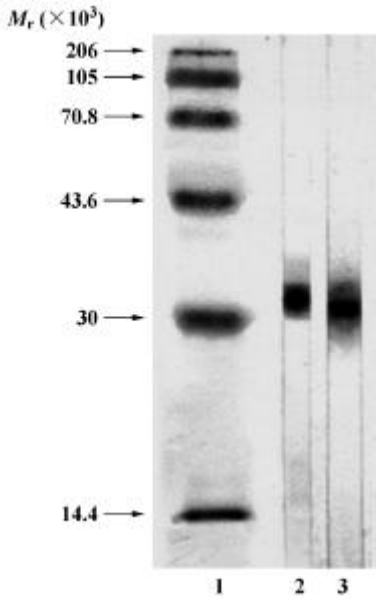

Fig 3. Identification of tryptase and chymase by specific monoclonal antibody AA_5 (against tryptase) and CC_1 (against chymase) by Western blotting. Lane 1: M_r markers, Lane 2: AA_5, Lane 3: CC_1.

effective in separating chymase from tryptase as well as other irrelevant proteins. Both chymase and tryptase activities were clearly separated from major protein peak (before fraction 6). Fractions 23 and 24 were highly purified tryptase, fraction 25 was a mixture of tryptase and chymase and fractions 26-28 were highly purified chymase. More than 55-fold increase in chymase pu-

rity was achieved by heparin affinity chromatography with approximately 32 % recovery of the chymase activity applied (Fig 4). A further 6.4-fold increase in specific chymase activity with an approximately 62 % recovery was obtained by S-200 Sephacryl gel filtration chromatography. As for heparin agarose, S-200 Sephacryl was able to clearly separate chymase from tryptase. The major protein peak was matched with tryptase peak, indicating that the majority of proteins in the sample applied were tryptase, whereas chymase activity was corresponded to the second protein peak (Fig 5). The final product showed only a single migration band of M_r 30 000 on 10 % SDS-PAGE (Fig 2B), and the band could be blotted by anti-chymase monoclonal antibody CC_1 (Fig 3).

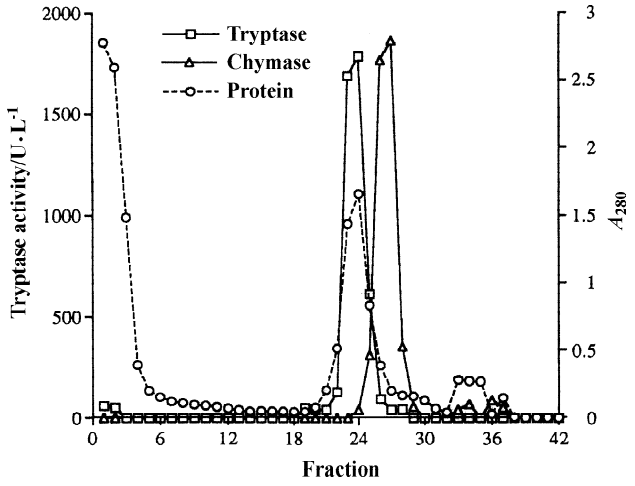

Fig 4. Isolation of chymase from human skin tissue by heparin affinity chromatography.

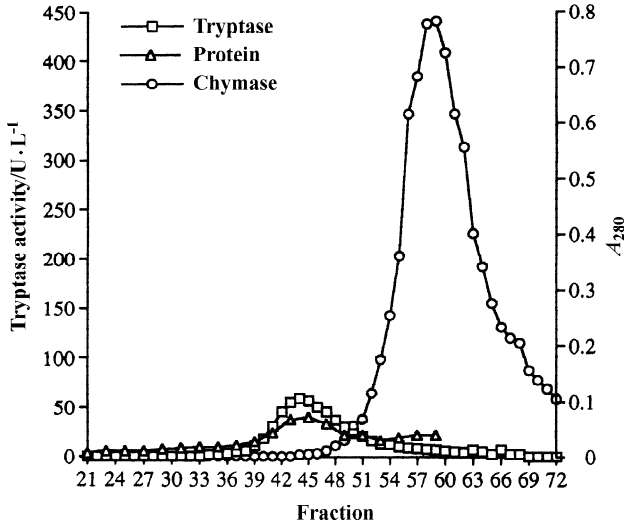

Fig 5. Separation of tryptase from chymase by S-200 Sephacryl gel filtration chromatography.

Among non-native proteinase inhibitors, *N*-(1-hydroxy-2-naphthoyl)-*L*-arginyl-*L*-prolinamide hydrochloride (HNAP, Axys, USA), leupeptin (Sigma), antipain (Sigma), benzamidine (Sigma), and protamine (Sigma) inhibited more than 90 % enzymatic activity of tryptase within 30-min incubation period. HNAP, leupeptin, and antipain had little inhibitory effects on enzymatic activity of chymase (Tab 1). In contrast, SBTI (Sigma), Z-Ile-Glu-Pro-Phe-CO_2Me (ZIGPPM, gift from Ferring Research Institute, UK), and chymostatin (Sigma) inhibited more than 95 % enzymatic activity of chymase within 30-min incubation period, but had no effect on tryptase activity (Tab 1).

Tab 1. Inhibition of enzymatic activity of tryptase or chymase towards synthetic substrates by non-native proteinase inhibitors.

Inhibitor	Concentration	Percentage inhibition of enzymatic activity/%	
		Tryptase (10 U/L)	Chymase (100 U/L)
None		0	0
HNAP	100 μmol· L^{-1}	102±4	0
Leupeptin	100 mg· L^{-1}	99.8±2.0	7±4
Antipain	100 mg· L^{-1}	99±3	18±4
Benzamidine	100 mg· L^{-1}	98.1±2.0	ND
Protamine	100 mg· L^{-1}	94±4	ND
Aprotinin	100 mg· L^{-1}	32±3	7±4
SBTI	100 mg· L^{-1}	0	99.2±2.0
ZIGPPM	1 μmol· L^{-1}	0	98.1±2.0
Chymostatin	10 mg· L^{-1}	0	95.0±0.3

BAPNA 2 mmol/L was employed as substrate for tryptase and SAAPP 0.7 mmol/L was used as substrate for chymase. All experiments were performed in triplicate. The enzyme was incubated with each inhibitor on ice for 30 min before addition of substrate solution. ND=not determined.

Native proteinase inhibitors α_1-antitrypsin (Sigma) and secretory leukocyte protease inhibitor (SLPI, R&D Systems, USA) inhibited more than 90 % enzymatic activity of chymase within 20-min incubation period. Extending incubation time to 40 or 80 min had little effect on the actions of these 2 inhibitors (Tab 2). On the other hand, lactoferrin appeared to enhance chymase enzymatic activity to a large degree, particularly after 20- and 80-min incubation periods (Tab 2). All three inhibitors had weak inhibitory actions on tryptase at 40

Tab 2. Inhibition of enzymatic activity of tryptase or chymase towards synthetic substrates by native proteinase inhibitors a_1-antitrypsin (a_1-AT), SLPI, and lactoferrin.

	Concentration /μmol· L^{-1}	Inhibition of enzymatic activity/% 20 min		40 min		80 min	
		Chymase	Tryptase	Chymase	Tryptase	Chymase	Tryptase
	None	0	0	0	0	0	0
α_1-AT	0.5	-29±9	-21±3	12±3	23.0±1.2	-67±17	6.7±1.7
	5.0	57.0±0.4	-23±3	74±3	3.8±0.9	32±5	-12.0±1.5
	50	93.0±1.3	-22.0±2.1	92.0±0.7	5.4±0.2	90±3	-11.0±0.6
SLPI	0.5	85.0±2.1	-14.0±2.4	89.0±0.6	30±1.0	76.0±1.9	13.0±1.7
	5.0	93.0±2.4	-8±3	96.0±1.3	14.0±0.9	89.0±1.2	7.2±1.5
	50	96.0±0.20	-8.8±0.8	98.0±1.9	23.0±0.5	92.0±0.20	15.0±1.1
Lactoferrin	0.5	-104±11	-20.0±1.5	-31±4	35.0±0.8	-138±10	-11.0±0.4
	5.0	-123±11	-27.0±1.7	-51±6	23.0±0.8	-176±12	1.4±0.6
	50	-121±3	-22.0±1.4	-47±9	7.4±0.7	-176±4	14.0±0.7

BAPNA 2 mmol/L was employed as substrate for tryptase and SAAPP 0.7 mmol/L was used as substrate for chymase. The concentration of tryptase or chymase was 10 U/L or 100 U/L, respectively. All experiments were performed in triplicate. The enzyme was incubated with α_1-antitrypsin, SLPI, or lactoferrin on ice for 20, 40, and 80 min before addition of substrate solution.

min incubation period.

DISCUSSION

NaCl 0.4 mmol/L in equilibration buffer of heparin affinity chromatography is crucial for obtaining high purity of tryptase as less than NaCl 0.2 mmol/L in equilibration buffer would not separate tryptase from brown and green protein and less than NaCl 0.3 mmol/L would not remove green protein in lung preparations. Minimum of 22 mg tryptase-containing protein could be obtained from approximately 500 g lung tissue by heparin affinity chromatography procedure. It is therefore, a most effective and repeatable chromatography method for isolation of tryptase from human tissue preparations.

However, heparin also binds to some other mediators or growth factors such as antithrombin III, heparin cofactor, protein C, and plasminogen activator inhibitor I under physiological conditions[21]. This suggested that there could be a small possibility that some unwanted proteins contaminate tryptase preparations. Since molecular weights of potential contaminated proteins are most likely smaller than M_r 50 000 and M_r of an intact tetrameric tryptase is 134 000[22], tryptase can be easily separated from these proteins by S-200 Sephacryl gel filtration chromatography.

It is more economical and less complicated than the procedures reported previously for isolation of human skin[23] or tonsil chymase[24]. It required less than NaCl 0.2 mol/L ionic strength to capture chymase in crude extract by heparin. To separate chymase from tryptase, a deep gradient of NaCl (0.4-2.0 mol/L) was applied to heparin agarose. The majority of chymase activity could be separated from tryptase activity by this method. This means that there are two major products chymase and tryptase generated from the purification procedure.

Based on the M_r difference, chymase (30 000) and tryptase (134 000)[19] were further separated by S-200 Sephacryl gel filtration chromatography procedure. At this stage, chymase was purified more than 350-fold, and should be useful for functional studies.

It is still at the early stage to develop a clinically effective drug against tryptase. Therefore, investigation of the actions of existing proteinase inhibitors on tryptase may accelerate the developing process. Among these non-native inhibitors, HNAP is a relatively specific inhibitor of tryptase[25], whereas leupeptin, antipain, benzamidine, and SBTI are known inhibitors of human trypsin. The property that enzymatic activity of tryptase could be inhibited by leupeptin, antipain, and benzamidine, but not by SBTI makes tryptase different from trypsin, of which enzymatic activity could be in-

hibited by SBTI. This suggested that an inhibitor drug of tryptase developed would only eliminate tryptase-induced inflammation, but not affect the protein digestion function of trypsin in the intestinal tract. The actions of HNAP, leupeptin, antipain, and benzamidine are most likely to block the catalytic sites of tryptase, therefore were able to abolish tryptase enzymatic activity towards BAPNA. Since chymostatin is an inhibitor of human chymotrypsin, its ability to inhibit the activity of chymase may reflect these two proteases sharing the similar inhibitory mechanism. Lack of inhibitory actions of aprotinin on either tryptase or chymase is not surprising as it is really an inhibitor of tissue kallikrein[26] and neutrophil cathepsin G[27]. The action of protamine on tryptase is to remove heparin from its tetrameric structure so that dissociated tryptase molecule loses its activity[28]. The incomplete inhibition of tryptase activity by protamine in the current study may reflect that the action of protamine on tryptase is a relatively slow process. ZIGPPM is a relatively specific inhibitor of human chymase[29], and its ability to inhibit 98 % chymase activity in the current study proved that it is also a potent inhibitor with a mixed inhibitory pattern[17]. Although the effects of some of the above non-native inhibitors on enzymatic activities of tryptase or chymase were previously tested these isolated individual reports would hardly compare with each other. The current study examined the actions of each protease inhibitor on enzymatic activities of both tryptase and chymase under the similar assay conditions. This parallel study will provide more reliable information for development of inhibitor drugs.

The actions of native protease inhibitors SLPI, α_1-antitrypsin, and lactoferrin on enzymatic activities of tryptase or chymase were also previously reported. However, most of these isolated individual reports either in different assay conditions or upon tryptase or chymase only. Under the similar assay conditions, it is interesting to learn that the 2 common protease inhibitors in man, SLPI and α_1-antitrypsin, are able to inhibit enzymatic activity of chymase, but not tryptase. The failure of inhibition of tryptase activity by SLPI was a finding different from the report[30]. This could result from the quantity of SLPI used in the current study was not sufficient enough to dissociate heparin from tryptase tetrameric complex[31]. The present work made it clearer that lactoferrin had little direct inhibitory actions on tryptase activity, but it could destabilize tryptase by replacing heparin[32], a stabiliser of tryptase[22]. It was surprised to observe that lactoferrin was capable of enhancing enzymatic activity of chymase. Since there was no added heparin in the assay the actions of lactoferrin on chymase were unlikely to associate with heparin[33]. The stimulatory action of lactoferrin on chymase activity being reduced at 40 min in comparison with 20 min and 80 min was hard to understand as little is known about the relationships between these two proteins. It may be related to the interchanges of spatial configurations between the two molecules. Obviously, more works are required to understand this novel issue.

The actions of native protease inhibitors may represent a self-regulation mechanism of protease-mediated inflammation in man. The imbalance between proteases and protease inhibitors could be the cause of certain diseases. As these native inhibitors are naturally originated from human being, human body should easily accept them when they are administered as clinical drugs. In conclusion, the parallel investigation of the actions of protease inhibitors on tryptase and chymase would provide more reliable and comparable information, which will be beneficial for the development of inhibitor drugs.

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