Activity-based mass spectrometric characterization of proteases and inhibitors in human saliva

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Abstract

Proteases present in oral fluid effectively modulate the structure and function of some salivary proteins and have been implicated in tissue destruction in oral disease. To identify the proteases operating in the oral environment, proteins in pooled whole saliva supernatant were separated by anion-exchange chromatography and individual fractions were analyzed for proteolytic activity by zymography using salivary histatins as the enzyme substrates. Protein bands displaying proteolytic activity were particularly prominent in the 50–75 kDa region. Individual bands were excised, in-gel trypsinized and subjected to LC/ESI-MS/MS. The data obtained were searched against human, oral microbial and protease databases. A total of 13 proteases were identified all of which were of mammalian origin. Proteases detected in multiple fractions with cleavage specificities toward arginine and lysine residues, were lactotransferrin, kallikrein-1, and human airway trypsin-like protease. Unexpectedly, ten protease inhibitors were co-identified suggesting they were associated with the proteases in the same fractions. The inhibitors found most frequently were alpha-2-macroglobulin-like protein 1, alpha-1-antitrypsin, and leukocyte elastase inhibitor. Regulation of oral fluid proteolysis is highly important given that an inbalance in such activities has been correlated to a variety of pathological conditions including oral cancer.

Keywords

Degradation; Enzymatic; Histatin; Oral; Proteolytic

1 Introduction

Human whole saliva (WS) is the fluid which bathes oral hard and soft tissues and its constituents are responsible for the maintenance of a healthy oral condition. WS is derived from different sources that are exocrine and non-exocrine in nature. The exocrine contributions, also called salivary glandular secretions, include the fluids from the parotid, submandibular, and sublingual glands. In addition, minor salivary glands are lined throughout most of the oral cavity, including Von Ebner's glands in circumvallate papillae of the tongue. The primary secretion flows through a network of ducts toward the oral cavity. Once the glandular secretions emanate into the oral environment, they are mixed with a number of non-exocrine components, which include gingival crevicular fluid, oral microorganisms, desquamated oral epithelial cells, other host and bacteria-derived components and dietary factors, ultimately forming WS.

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The proteinaceous elements that are functionally responsible for oral health are expected to be effective in WS which contains a mixture of enzymes. These enzymes, which can be derived from either of the various sources eluded to above, have been described to proteolytically degrade some salivary proteins with high efficiency. Hence, oral fluid proteolysis is an important factor to be taken into account in saliva protein structure–function analyses and in diagnostic exploitations. Bacterial as well as human-derived enzymes have also been implicated in the destruction of oral tissues leading to pathological conditions. Approaches exploring specific oral enzyme activities have shown that hydrolysis of benzoyl-arginine naphthylamide in saliva correlated with the numbers of spirochetes in subgingival plaque samples, and to be an indicator of periodontal disease [1]. The role of collagenases, in particular matrix metalloproteinase-8 (MMP-8), have been well established in tissue destruction associated with periodontitis and implantitis [2,3]. Additional investigations focusing on the role of oral enzymes in malignant transformations have implicated polymorphism in the gene expression of MMP-13 with a highly aggressive form of oral cancer [4]. Despite the evidence of a relationship between oral proteolytic activities and oral health and disease, the identities of most enzymes operating in the oral cavity are elusive.

One group of salivary proteins that is exceptionally susceptible to WS proteolysis is human histatins [5–9]. Histatins are non-glycosylated small histidine-rich salivary proteins. Structural analysis of fragments derived from histatins and other peptides in WS has provided a first, albeit indirect, insight into the saliva-associated enzymatic cleavage specificities. The actual proteases responsible for the degradation of histatins and other salivary proteins in the oral cavity are essentially unknown. The present study employed a histatin zymography approach in conjunction with LC-ESI-MS/MS to achieve the first global activity-based identification and characterization of proteolytic enzymes in oral fluid.

2 Materials and methods

2.1 WS collection

Stimulated WS was obtained from five orally healthy subjects. Prior to participation, all subjects consented to participation in the study according to approved protocols of the Institutional Review Board at Boston University Medical Center. WS (5 mL) was collected between 10 and 11 a.m. at least 1 h after the last meal. WS was stimulated using 1 g of paraffin wax for mastication and was collected by expectoration (Parafilm, American National Can™, Chicago, IL). Immediately after collection WS was centrifuged at $14000 \times g$ for 20 min at 4° C, and the resultant WS supernatant (WSS) from each of the five subjects was pooled.

2.2 Anion-exchange chromatography

An aliquot of 15 mL of pooled WSS was dialyzed against milliQ water for 16 h. The sample was loaded on a strong anionic-exchange column (MonoQ™ HR16/10; Amersham Biosciences, Piscataway, NJ) connected to a fast protein liquid chromatography (FPLC) (Pharmacia Biotech, Piscataway, NJ). Proteins were eluted at a flow rate of 2 mL/min using buffer A (50 mM NaCl, 50 mM Tris-HCl, pH 8.0) and buffer B (1 M NaCl, 50 mM Tris-HCl, pH 8.0) applying the following gradient scheme: 0–45 min, 0% buffer B; 46–275 min, 0–25% buffer B; 276–300 min, 25–40% buffer B; 301–325 min, 40–100% buffer B; 325–355 min, 100% buffer B; 355–370 min, 100–0% buffer B. The absorbance was monitored at 214 nm and the eluate was collected in 10 mL aliquots. Fractions were dialyzed against water using dialysis tubing with a molecular weight cut-off of 1000 and lyophilized (Virtis, Gardiner, NJ). The dried 10 mL sample was dissolved in 1 mL of milliQ water for further analysis.

2.3 Synthetic histatins

Synthetic histatins 1, 3, and 5 were obtained from commercial sources (Quality Controlled Biochemicals, Hopkinton, MA; American Peptide Company, Sunnyvale, CA). Proteins were dissolved in milliQ water to approximately 10 mg/mL and their precise concentrations were determined by measuring the absorbance at 215 nm using a specific absorption coefficient (ε) of 20 mL mg^{-1} cm^{-1}.

2.4 Cationic PAGE

WS and WSS sample aliquots (100 µL) to be analyzed by cationic PAGE were dried using a Vacufuge concentrator (Eppendorf Westbury, NY) and resuspended in sample solution containing 0.04% methyl green (Thermo Fisher, Waltham, MA) in 40% sucrose (Sigma, St. Louis, MO). Cationic PAGE was carried out as previously described [10,11]. Gel polymerization was accelerated by exposing the gels to a light source (60 Watts). Gels were stained with Coomassie Brilliant Blue R-250 (0.1% v/v) in 10% v/v acetic acid and 40% v/v methanol, and destained in the same solution not containing the dye.

2.5 Zymography

WS, WSS and anion-exchange fractions of WSS (100 µL aliquots) were dried and subjected to zymography. Zymogram analysis was performed essentially as described [12] with the modification that synthetic histatin 1, 3, or 5 were used as enzyme substrates and incorporated in the separating gel mixture. Proteins in WS and WSS were separated by electrophoresis. The separating gel mixture contained 0.1% w/v histatin 1, 3, or 5, 8% w/v acrylamide, 0.4% w/v bisacrylamide, 0.1% w/v SDS and 375 mM Tris-HCl, pH 8.8, and the stacking gel contained 4% w/v acrylamide, 0.2% w/v bisacrylamide, 0.1% w/v SDS, and 126 mM Tris-HCl, pH 6.8. Polymerization was initiated upon the addition of 0.05% w/v ammonium persulfate and 0.1% v/v TEMED. Before loading onto the gel, samples to be analyzed (WSS, WS or anion-exchange fractions) were dried by a Vacufuge concentrator (Eppendorf) and mixed with 20 µL zymogram sample buffer containing 25% glycerol, 4% SDS, 0.01% Bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8. The running buffer contained 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3. Gel electrophoresis was carried out at a constant voltage of 120 V for 1 h at 4°C. After electrophoresis, enzymatic conversion of the gel-incorporated histatin proteins was facilitated by renaturing of the gel for 30 min at room temperature in 2.5% Triton X-100 in milliQ water. Gels were washed in buffer containing 20 mM Tris-HCl and 1 mM CaCl$_2$, pH 7.5, and developed for 16 h at 37°C in the same buffer. Staining of the gel was achieved in 0.1% w/v Coomassie Brilliant Blue R-250 in 10% v/v acetic acid and 40% v/v methanol, and destaining was carried out by boiling the gel in milliQ water for 8 min to visualize the histatin-degrading proteases.

2.6 In-gel trypsinization of zymogram bands

After zymography, the bands displaying enzymatic activities were excised and cut into 1 mm^3 pieces using a new razor blade for each band. The gel pieces were destained in washing buffer (50% ACN in 100 mM ammonium bicarbonate) for 16 h, followed by removal of ACN, dehydration of the gel pieces in 100% ACN, and drying in a vacuum concentrator (Speedvac, Millipore). A 50 µL aliquot of digestion solution containing 12.5 µg/mL modified sequencing-grade trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate (pH 8.0), was added followed by incubation at 37°C for 16 h. Peptides were recovered from the supernatant after washing the gel pieces with solution containing 50% ACN and 5% acetic acid. The extracts were dried in a speed-vac and the samples were then stored at 4°C until analysis.

2.7 LC-ESI-MS/MS

The dried, in-gel zymogram digests were reconstituted in 5–10 µL of solvent A containing 2.5% ACN and 0.1% formic acid. Samples were applied to a nano-scale RP-HPLC capillary column (75 µm inner diameter × ~10 cm length) which was created in-house by packing 5 µm C18 spherical silica beads (Micron Bioresource, Auburn, CA) into a fused-silica capillary with a flame-drawn tip. After equilibrating the column, each sample (3 µL) was loaded *via* a Famos auto sampler (LC Packings, San Francisco CA) onto the column. Peptides were eluted with a linear gradient of increasing buffer B (97.5% ACN, 0.1% formic acid) from 0 to 100% over a 15 min time interval followed by ESI (LTQ linear ion-trap mass spectrometer; ThermoFisher, San Jose, CA) yielding tandem mass spectra of specific fragment ions for each peptide.

2.8 Mass spectrometric data analysis

The MS/MS data were searched against three main databases: (i) the human protein database (Swiss-Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://expasy.org/sprot), (ii) a database containing all proteases known to date across the plant and animal kingdom ([13] MEROPS, http://merops.sanger.ac.uk/), and (iii) an oral microbial database consisting of all fully and partly sequenced oral microbial organisms to date (http://www.HOMD.org). In addition, the data were searched against databases of each of the microbial species individually to avoid the assignment of two peptides from one protein homologue to different microbial species. Searches were performed essentially as described [14] using Bioworks software (version 3.3.1) with the following parameters selected: (i) fully tryptic fragment; (ii) Delta CN$\geq$0.1; (iii) peptide probability$\leq$0.5, XCorr score$\geq$2.0 and 3.5 for $z = 2$ and 3, respectively. All spectra were manually examined for the presence of at least three consecutive b- and y-ions. Proteins of which at least two different *bona fide* peptides were identified were considered a confident match. Searches of the MS/MS data against the human database with sequences in reversed order yielded 0.25% false positive identifications by protein among which there were no proteases.

3 Results

3.1 Comparison of protease activities in WS and WSS

To determine the distribution of WS enzymatic activities in the particulate and soluble fraction of WS, histatin 5 was added to WS and WSS and its degradation was followed as a function of time (Fig. 1). Natural histatin 5 levels in WS and WSS (right lanes) were negligible as compared to the added amount of histatin 5 (left lanes). Histatin disappearance was quantitated by densitometric analysis of the histatin band at each of the sampling time points (Fig. 1C). From the densitometric plots, it could be established that the half-life of the disappearance of histatin 5 was 8.7 min in WS and 65.3 min in WSS. From these data, it can be estimated that the degradation rates in WS are approximately 7.5 times faster than in WSS, and that 88.2% of the proteolytic activity in WS is associated with the non-soluble components.

3.2 Comparison of protease profiles in WS and WSS

To gain insight into the approximate molecular weights of the histatin-degrading proteases, zymography was carried out with histatin 1, 3, or 5 copolymerized in the separating gel (Fig. 2). Different volumes of WS and WSS were loaded on the gel to compensate for the higher overall proteolytic activity of WS as compared with WSS enabling a comparison of the protease banding patterns. WS and WSS generally displayed similar protease activity patterns toward all three histatins, although higher activities were observed toward histatin 3 and 5 as compared with histatin 1 (Fig. 2). This corroborates with earlier reports that histatin 1 is more resistant to proteolytic breakdown in saliva than histatins 3 and 5 [8,15]. Multiple protease bands were noted in the 50–75 kDa molecular weight region. Based on the qualitative similarities in the

zymogram patterns of WS and WSS, WSS was further utilized for the isolation of the proteolytic enzymes.

3.3 Fractionation of WSS by FPLC anion-exchange chromatography

To reduce the complexity in the WSS sample, WSS proteins were dialyzed and fractionated by anion-exchange chromatography (monoQ). The choice for anion-exchange resin material was based on a preliminary experiment in which WSS was incubated with DEAE resin material (anionexchange) followed by batch-wise elution with increasing concentrations of NaCl. It was noticed that very little activity was recovered in the void volume, indicating that the enzymes targeting histatins were predominantly negatively charged. Proteins were eluted from the monoQ column using a gradient from 50 to 100 mM NaCl in 50 mM Tris-HCl (pH 8) (data not shown). The bound (anionic) proteins eluted primarily as three major and two minor peaks. Nevertheless, all 65 eluting fractions were collected for protease activity evaluation.

3.4 Protease activity in anion-exchange fractions

All 65 FPLC fractions were desalted and concentrated tenfold. Protease activity in each fraction was assessed by adding a mixture of histatins 1, 3, and 5 and monitoring their degradation over a 90 min time interval by cationic PAGE (Fig. 3). The selected 90 min time interval stems from results presented in Fig. 1 and allowed for a relative comparison in activities between fractions. Based on the degradation results obtained, the fractions could be divided into three different groups. A first group comprising fractions 20, 21, 25, 26, 31, 36, 48, and 49, displayed a generalized activity toward all three histatins, noted by the disappearance of all three proteins over time and the concomitant appearance of smaller molecular weight fragments (Fig. 3A). Proteases in the second group, which included fractions 6, 9, 10, and 14 to 18, appeared to selectively target histatin 3, given the relative stability of the histatin 1 and histatin 5 bands (Fig. 3B). The remainder of the fractions exhibited no or negligible proteolytic activity toward any of the histatins (Fig. 3C). The thirteen most active fractions, eight from the first group and five from the second group, were chosen for further analysis by histatin zymography.

3.5 Zymography of FPLC fractions

The eight WSS fractions with activity toward all histatins (20, 21, 25, 26, 31, 36, 48, and 49) were subjected to histatin 5 zymography, and the five fractions that appeared selectively active toward histatin 3 (6, 9, 14, 15, and 17) were analyzed by histatin 3 zymography (Fig. 4A). As intended, the complexity of the protease patterns in most fractions were strongly reduced as compared with the patterns observed in unfractionated WSS. Most of the protease bands resided in the 50–75 kDa region, as seen in WSS. The major protein bands exhibiting proteolytic activity were excised and numbered (Fig. 4B). Only in the case of fraction 14, the zymogram bands were very weak. However, bands with electrophoretic mobilities comparable to those in fraction 14 but with higher intensity were observed in other fractions and excised. In some cases, protein bands from different fractions showing similar electrophoretic mobilities were pooled. In total, 16 protease-containing gel pieces were further processed.

3.6 Mass spectrometric identification of proteases and protease inhibitors in WSS

Proteins in the 16 protein bands were in-gel trypsinized and the peptides extracted and sequenced by LC-ESI-MS/MS. Unexpectedly, searches against the oral microbial databases yielded only four reliable protein identifications among which there was no protease. In a control experiment, a digest of *P. gingivalis* culture supernatant searched against its respective database yielded the identification of multiple enzymes including the potent proteolytic enzymes gingipain R and gingipain K (data not shown). The results of the searches against the human database are summarized in the Supporting Information Table, which summarizes all proteins identified, and in Table 1 containing the proteases and inhibitors only. Interestingly,

in most bands, proteases as well as inhibitors were identified. Some proteases such as kallikrein 1 appeared in multiple zymogram bands. Likewise, some inhibitors such as alpha-1-antitrypsin, were detected in more than one band. Additional characteristics of the proteases and inhibitors identified are listed in Table 2 and Table 3, respectively. Of the 13 proteases found, six belonged to the family of serine proteases (lactotransferrin, myeloblastin, transmembrane protease serine 11D, dipeptidylpeptidase 2, furin and kallikrein-1). The remaining seven proteases included three cysteine proteases, three metallo proteases and one aspartyl protease. Also listed in Table 2 are the preferred cleavage site specificities of each of the enzymes. The serine proteases lactotransferrin, transmembrane protease serine 11D, furin and kallikrein-1 exhibit preferential cleavage specificity after an R or K residue. Of the ten protease inhibitors identified, alpha-2-macroglobulin-like protein 1 (α2ML1), alpha-1-antitrypsin, and leukocyte elastase inhibitor were found most frequently, each displaying inhibitory activities toward various classes of enzymes (Table 3). No additional proteases/inhibitors were identified when the MS/MS data were searched against the designated MEROPS database.

4 Discussion

The present work aimed to characterize active enzymes in oral fluid. Before this study, the proteases lactotransferrin, kallikrein-1, dipeptidyl-peptidase and cathepsins [16–22], and the inhibitors alpha-1-antitrypsin, cystatin SA and metalloproteinase inhibitor 1 [23–25] had been detected in saliva and/or gingival fluid by a variety of classical, non-MS-based techniques. In fact, with the exception of alpha-1-antichymotrypsin and kininogen-1, all enzymes and inhibitors listed in Table 1 and Table 2 have been reported recently to be saliva constituents following extensive proteomic analysis of individual salivary secretions [9,26–30]. In the present investigation, we employed a zymography approach in conjunction with LC-ESI MS/MS. Therefore, the protease identifications made were not only based on mass spectrometric detection (presence), but also on actual activity of the enzyme. Furthermore, different from typical casein or gelatin zymography approaches, histatin zymography facilitated the identification of proteases with activity toward a salivary protein substrate.

4.1 Protease-inhibitor associations

One of the most intriguing and unexpected findings in this exploration was the presence of proteases and inhibitors in multiple bands on the zymogram gel. Evidence for the existence of various isoforms of some proteases have been reported. For instance, kallikrein-1 is variably and extensively post-translationally modified yielding molecular masses of <20, 45, 60, 90 and >200 kDa [31]. Also lactotransferrin has been reported to exist in multiple isoforms [32]. As for most proteases, the majority of the inhibitors appeared in multiple bands. Noticeably, in some cases, the anticipated molecular weight of a protease–inhibitor couple in a particular band on the zymogram did not match the theoretical molecular weights of the identified constituents. For instance, the active component in band 15 migrates with an approximate molecular weight between 25 and 37 kDa, whereas the theoretical molecular weights of its constituents, leukotriene A4 hydrolase and alpha-1-antitrypsin, each exceed this molecular weight. Although fragmentation of the protease and inhibitor could perhaps account for their appearance in the low molecular weight regions, some caution is to be exerted in the interpretation of true protease–inhibitor associations as opposed to coincident identifications.

4.2 Mammalian proteases in WSS

Thirteen proteases from mammalian origin were found upon mass spectrometric processing of the active bands. Three of these proteases, lactotransferrin, kallikrein-1, and human airway trypsin-like protease (HAT) (transmembrane protease, serine 11D) likely play a dominant role in histatin proteolysis, because they are serine proteases exhibiting preferential cleavage specificities C-terminal to arginine (R) and lysine (K) residues, amino acids which are enriched

in histatins. Iron-free lactotransferrin cleaves the enzyme substrate Z-YR-AMC, a substrate showing homology to the RGYR template, which is present and targeted in histatin 3 [5]. Because lactotransferrin in human saliva contains very low iron levels ($0.0212\pm0.0081\%$; [33]), lactotransferrin is predominantly present in the active apo form, which would allow it to function as a protease in oral fluid. Like lactotransferrin, kallikrein-1 is a trypsin-like serine protease with preference for arginine at the P1 position. Its activity has been reported to be inhibited by PMSF, benzamidine, leupeptin, kallistatin, protein C inhibitor, and aprotinin [34], some of which in combination are highly effectively in preventing histatin proteolysis in WSS (Sun *et al*., unpublished data). Evidence for the likelihood for the involvement of kallikrein-1 in histatin degradation in the oral cavity stems from a recent *in vitro* study showing that histatin 5 was cleaved by kallikrein-1 C-terminal to R^{10}, K^{11}, and R^{12} [35]. Another prominent protease identified was transmembrane protease, serine 11D which is also named HAT [36]. HAT protein is expressed in the cells of submucosal serous glands of human bronchi and trachea [36] and it has been detected in human glandular secretion and in WS [26,29]. The present study unequivocally identifies HAT to be functional in the oral cavity.

4.3 Bacterial proteases in WSS

A body of evidence suggests that a likely source for WS proteases are oral microorganisms [37–39]. Consistent with this, most of the enzymatic activity was found to be associated with the particulate component of WS, of which bacteria are a prominent constituent. Unexpectedly, when the MS/MS data were queried against bacterial databases, a very low number of proteins were identified. Similar low identifications of bacteria-derived proteins in WSS have been reported by other investigators [40]. Frequently, only one peptide was detected from a protein, which did not meet our criteria for protein identification. Given the high probability that oral microbial proteases are at least in part responsible for salivary protein digestion, it is anticipated that further sequencing of oral microbial genomes will enable identifications of bacterial proteases in WS in the near future and expand the numbers of saliva-related protease identifications.

4.4 Protease inhibitors in WSS

Interestingly, we found a relatively large number of protease inhibitors in the zymogram bands. Their identification by multiple peptides suggests that these inhibitors were enriched in the processed zymogram bands. Noticeably, α2ML1, alpha-1-antitrypsin and leukocyte elastase inhibitors, all exhibiting inhibitory activities toward various classes of enzymes, were observed most frequently. The broad-spectrum inhibitory activities of these inhibitors and their association with different proteases and/or isoforms of the same protease could explain their appearance in multiple bands in the zymogram gel. α2ML1 belongs to the αM super family comprising both protease inhibitors and components of the complement system [41]. The ability of αM proteins to inhibit proteases depends on its unique mechanism of inhibition by physical hindrance called the "trap mechanism" [42]. In this mechanism, entrapped proteases lose their ability to cleave high molecular weight substrates but would retain their capacity to cleave smaller molecular weight substrates [43,44] such as histatin 5. A similar "trap" mechanism of inhibition has been postulated for alpha-1-antitrypsin [45]. Alpha-1-antitrypsin, also named alpha-1 protease inhibitor and alpha-1-antiproteinase, is a serine protease inhibitor belonging to the Serpin family which inhibits a variety of proteases including elastate-1, elastate-2, thrombin, plasmin, cathepsin G, proteinase 3, chymotrypsin A, and kallikrein-related peptidase 4 [46]. Another member of the serpin family, leukocyte elastase inhibitor, was also frequently found in our analyses. It is known to be one of the most efficient protease inhibitors of neutrophil serine proteases including elastase, cathepsin G and proteinase-3 [47–49], and together with α2ML1 and alpha-1-antitrypsin represents an important component of the arsenal of inhibitors in saliva.

Overall, using pure histatins as substrates, the activity-based characterizations have not only enabled the identification of proteases effective in degrading histatins, but also led to the detection of an almost equal number of mammalian-derived protease inhibitors, many with broad-spectrum inhibitory activities. Their identification with high confidence in multiple zymogram bands suggests that they play a crucial role in modulating protease activity in the oral environment. Regulation of such activities is indeed important, given the evidence that uncontrolled proteolytic activities are at the basis of multiple pathological conditions, including oral and duodenal cancer [50–52]. A full understanding of the interplay of these proteases and inhibitors in oral as well as gastro-intestinal physiology would represent a novel area of investigation. Our data suggest that α2ML1, alpha-1 antitrypsin and leukocyte elastase inhibitor may play crucial roles in such regulatory processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

α2ML1	alpha-2-macroglobulin-like protein 1
FPLC	fast protein liquid chromatography
HAT	human airway trypsin-like protease
WS	whole saliva
WSS	whole saliva supernatant

Acknowledgments

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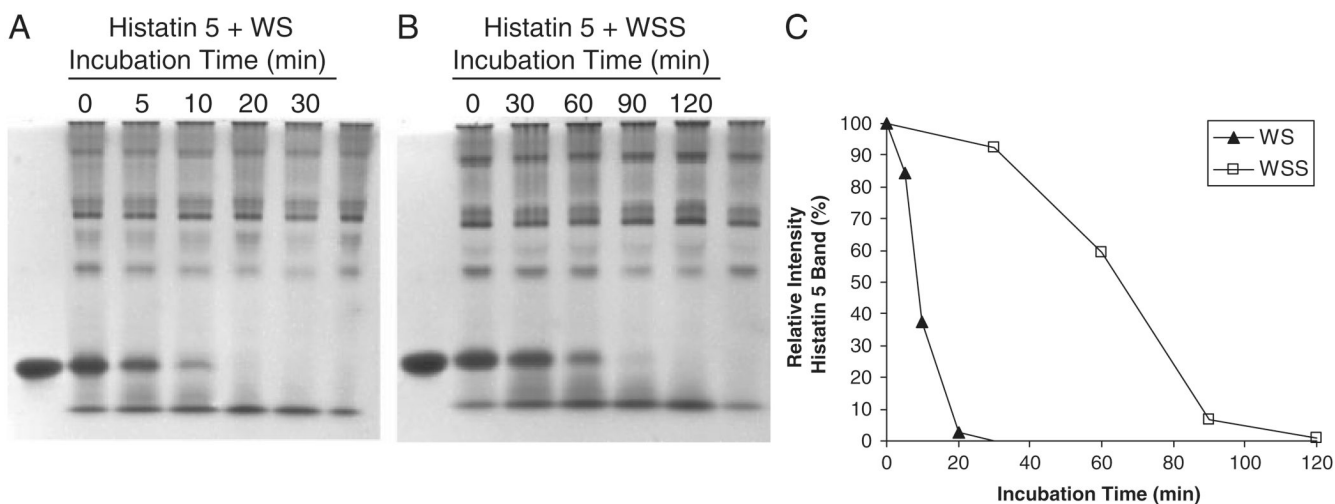
Figure 1.
Comparison of protease activities in WSS and WS. Synthetic histatin 5 (100 µg) was added to 1 mL of WS (A) or WSS (B) and incubated at 37°C. Aliquots of 100 µL were removed after the indicated time intervals, boiled, dried, and analyzed by cationic-PAGE. Left lanes in (A) and (B): 10 µg histatin 5; right lanes in (A) and (B): 100 µL WS and WSS, respectively. (C) densitometric analysis of histatin 5 as a function of incubation time in WS and WSS. The intensity of the histatin 5 band immediately upon addition to WS or WSS ($t = 0$) was set to 100%.
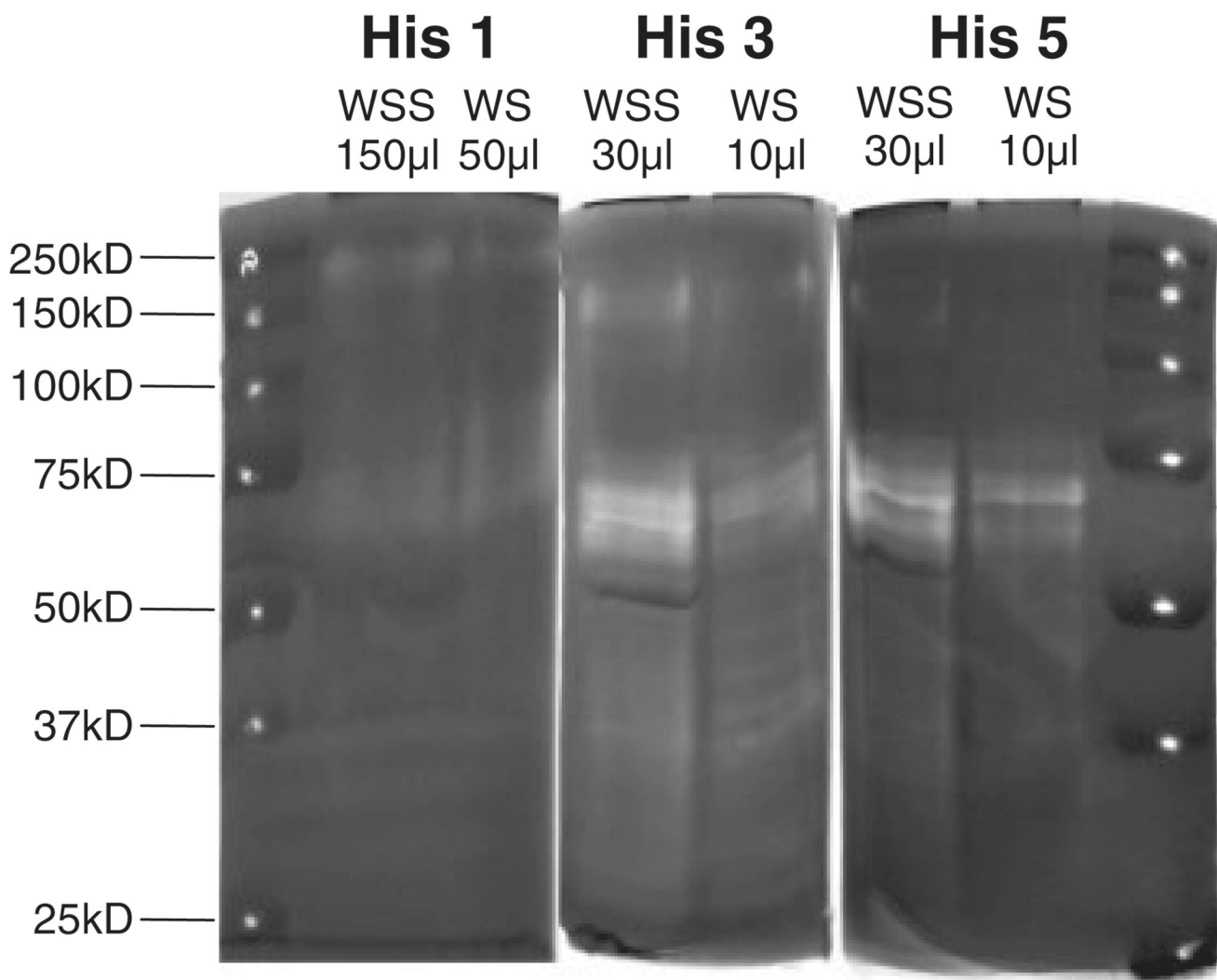
Figure 2.
Histatin zymography of WSS and WS. WSS and WS samples were analyzed on zymogram gels with incorporated histatin 1 (left panel), histatin 3 (middle panel) or histatin 5 (right panel). The volumes loaded of each of the saliva sample are indicated. The position of the molecular weight standards (pierced) are depicted in the far left and right lanes.
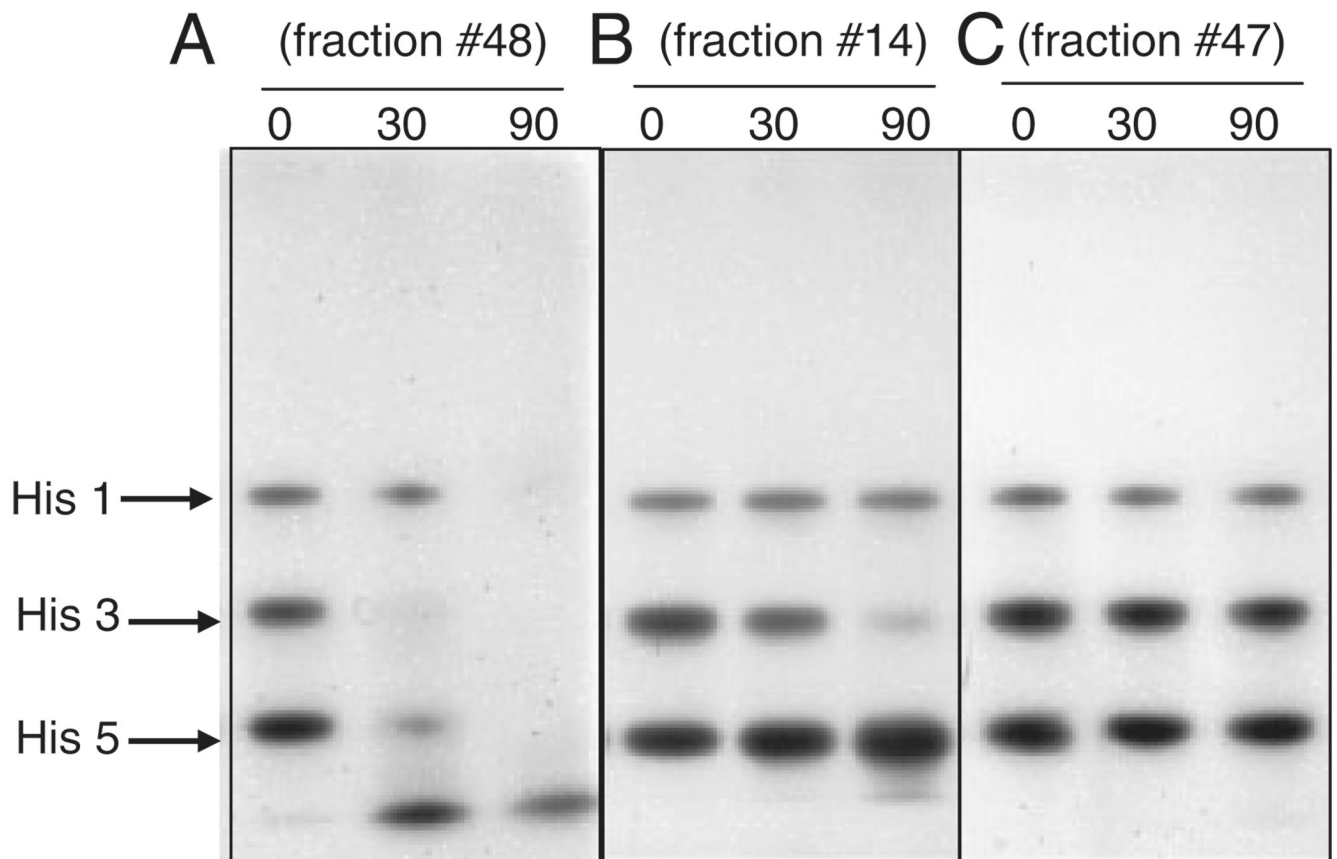
Figure 3.
Assessment of histatin-degradating activity in FPLC fractions from WSS. A mixture of synthetic histatin 1, 3, and 5 (5 µg each) was added to 161 µL of individual FPLC fractions of WSS proteins. After 0, 30, and 90 min of incubation, 50 µL aliquots were boiled, dried, and analyzed by cationic PAGE. (A) example of a fraction displaying activity toward all histatins, (B) example of a fraction displaying activity toward histatin 3 only. (C) example of a fraction devoid of proteolytic activity toward any of the histatins.
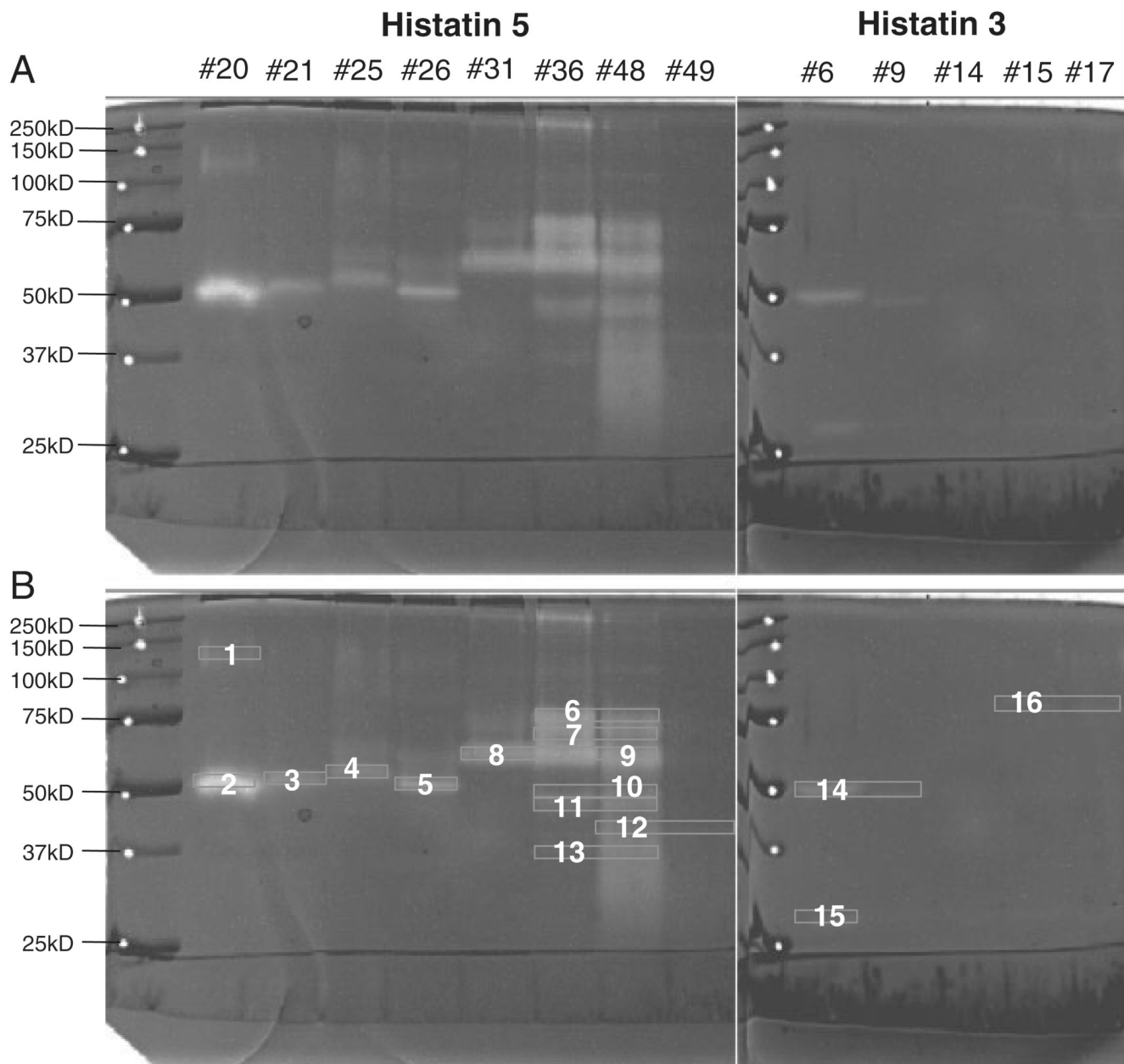
Figure 4.
Zymography of active WSS fractions. Proteins in fractions 20, 21, 25, 26, 31, 36, 48, and 49 were analyzed by histatin 5 zymography (left panel) and proteins in fractions 6, 9, 14, 15, and 17 were subjected to histatin 3 zymography (right panel). Left lanes, molecular weight standard. Sixteen protein bands displaying proteolytic activity were excised from the gel for subsequent trypsinization and processing by LC-ESI-MS/MS.

Table 1

LC-ESI-MS/MS characterization of proteases and inhibitors

Band ID	Total no. proteins identified	Protease	Entry name	Accession no.	No. of peptides
1	12	Lactotransferrin	TRFL_HUMAN	P02788	7
		Myeloblastin	PRTN3_HUMAN	P24158	2
		Alpha-2-macroglobulin-like protein 1	A2ML1_HUMAN	Q6ZW52	41
2	22	Dipeptidyl-peptidase 1	CATC_HUMAN	P53634	3
		Lactotransferrin	TRFL_HUMAN	P02788	3
		Leukotriene A-4 hydrolase	LKHA4_HUMAN	P09960	3
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	15
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	11
3	14	Alpha-1-antitrypsin	A1AT_HUMAN	P01009	5
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	3
4	34	Lactotransferrin	TRFL_HUMAN	P02788	19
		Dipeptidyl-peptidase 2	DPP2_HUMAN	Q9UHL4	3
		Dipeptidyl-peptidase 1	CATC_HUMAN	P53634	2
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	25
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	3
		Plasma protease C1 inhibitor	IC1_HUMAN	P05155	3
		Alpha-1-antichymotrypsin	AACT_HUMAN	P01011	3
		Alpha-2-macroglobulin-like protein 1	A2ML1_HUMAN	Q6ZW52	2
5	27	Lactotransferrin	TRFL_HUMAN	P02788	2
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	14
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	3

Band ID	Total no. proteins identified	Protease	Entry name	Accession no.	No. of peptides
		Cystatin-SA	CYTT_HUMAN	P09228	2
6	43	Lactotransferrin	TRFL_HUMAN	P02788	17
		Furin	FURIN_HUMAN	P09958	3
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	12
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	11
		Alpha-1-antichymotrypsin	AACT_HUMAN	P01011	7
		Angiotensinogen	ANGT_HUMAN	P01019	3
		Alpha-2-macroglobulin-like protein 1	A2ML1_HUMAN	Q6ZW52	2
		Kininogen-1	KNG1_HUMAN	P01042	2
7	37	Kallikrein-1	KLK1_HUMAN	P06870	3
		Angiotensinogen	ANGT_HUMAN	P01019	5
		Neuroserpin	NEUS_HUMAN	Q99574	3
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	2
8	30	Cathepsin D	CATD_HUMAN	P07339	8
		Kallikrein-1	KLK1_HUMAN	P06870	2
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	6
		Neuroserpin	NEUS_HUMAN	Q99574	6
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	2
9	28	Lactotransferrin	TRFL_HUMAN	P02788	16
		Carboxypeptidase E	CBPE_HUMAN	P16870	3
		Puromycin-sensitive aminopeptidase	PSA_HUMAN	P55786	3
10	11	Cathepsin L1	CATL1_HUMAN	P07711	5
		Kallikrein-1	KLK1_HUMAN	P06870	2
11	18	Puromycin-sensitive aminopeptidase	PSA_HUMAN	P55786	3

Band ID	Total no. proteins identified	Protease	Entry name	Accession no.	No. of peptides
		Kallikrein-1	KLK1_HUMAN	P06870	3
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	2
12	47	Puromycin-sensitive aminopeptidase	PSA_HUMAN	P55786	6
		Calpain-1 catalytic subunit	CAN1_HUMAN	P07384	5
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	3
		Plasma protease C1 inhibitor	IC1_HUMAN	P05155	3
13	31	Lactotransferrin	TRFL_HUMAN	P02788	17
14	18	Cathepsin D	CATD_HUMAN	P07339	3
		Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	3
15	14	Leukotriene A-4 hydrolase	LKHA4_HUMAN	P09960	3
		Alpha-1-antitrypsin	A1AT_HUMAN	P01009	2
16	15	Cathepsin D	CATD_HUMAN	P07339	4
		Transmembrane protease, serine 11D	TM11D_HUMAN	O60235	2
		Metalloproteinase inhibitor 1	TIMP1_HUMAN	P01033	2

Table 2

Characteristics of proteases identified by LC-ESI-MS/MS

Protease	Accession no.	Type	Cleavage site specificity[a]	Band ID
Lactotransferrin	P02788	Serine	−/−/−/rk+−/−/−/−	1, 2, 4, 5, 6, 9, 13
Myeloblastin	P24158	Serine	−/−/−/vat+−/−/−/−	1
Transmembrane protease, serine 11D	O60235	Serine	−/−/−/r+−/−/−/−	16
Dipeptidyl-peptidase 2	Q9UHL4	Serine	−/−/−/Pam+−/−/−/−	4
Furin	P09958	Serine	R/k/KR/R+sa/−/−/−	6
Kallikrein-1	P06870	Serine	−/−/−/R+−/−/−/−	7, 8, 10, 11
Dipeptidyl-peptidase 1	P53634	Cysteine	−/esfr+−/−/−/−	2, 4
Cathepsin L1	P07711	Cysteine	−/−/lfy/−+−/−/−/−	10
Calpain-1 catalytic subunit	P07384	Cysteine	−/−/l/−+a/−/p/−	12
Leukotriene A-4 hydrolase	P09960	Metallo	−/−/−/arpy+−/−/−/−	2, 15
Carboxypeptidase E	P16870	Metallo	−/−/−/ap+r	9
Puromycin-sensitive aminopeptidase	P55786	Metallo	−/−/−/lar+l/−/−/−	9, 11, 12
Cathepsin D	P07339	Aspartic	−/−/−/lf+−/−/−/−	8, 14, 16

[a] Nomenclature of cleavage site specificity used was as reported (http://merops.sanger.ac.uk/). Each cleavage site specificity is illustrated by eight amino acid residues (P4-P3-P2-P1-P1′-P2′-P3′-P4′) around the scissile bond indicated by the symbol +. Amino acids identified with high confidence at the indicated position are capitalized; amino acids identified with lower confidence are indicated in lower case; a dash indicates that the amino acid required at that position has not yet been established.

Table 3

Characteristics of protease inhibitors identified by LC-ESI-MS/MS

Protease inhibitor	Accession no.	Target enzyme[a]	Band ID
Alpha-2-macroglobulin-like protein 1	Q6ZW52	Chymotrypsin, subtilisin A, papain, thermolysin	1, 4, 6
Alpha-1-antitrypsin	P01009	Elastase-1, elastase-2, cathepsin G, thrombin, plasmin, kallikrein-related peptidase 4	2, 3, 4, 5, 6, 8, 11, 12, 15
Leukocyte elastase inhibitor	P30740	Elastase-2, cathepsin G, myeloblastin	2, 3, 4, 5, 6, 7, 8, 14
Plasma protease C1 inhibitor	P05155	Complement component activated C1r and C1s	4, 12
Alpha-1-antichymotrypsin	P01011	Kallikrein-related peptidase 14, cathepsin G	4, 6
Cystatin-SA	P09228	Cysteine protease	5
Angiotensinogen	P01019	Unknown	6, 7
Kininogen-1	P01042	Cysteine proteases (cathepsin L)	6
Neuroserpin	Q99574	uPA, tPA, plasmin	7, 8
Metalloproteinase inhibitor 1	P01033	MMPs	16

[a]Based on information available at the MEROPS website (http://merops.sanger.ac.uk/).