Fluorescent Probes for Studying Thioamide Positional Effects on Proteolysis Reveal Insight into Resistance to Cysteine Proteases

Chunxiao Liu,[a][b] Taylor M. Barrett,[a] Xing Chen,[a] John J. Ferrie,[a] and E. James Petersson*[a]

Dedication ((optional))

Abstract: Thioamide substitutions of the peptide backbone have been shown to reduce proteolytic degradation, and this property can be used to generate competitive protease inhibitors and to stabilize peptides toward degradation *in vivo*. Here, we present a straightforward sensor design that allows a systematic study of the positional effects of thioamide substitution using real-time fluorescence. Thioamide scanning in peptide substrates of five papain family cysteine proteases demonstrates that a thioamide at or near the scissile bond can slow proteolysis in all cases, but that the magnitude of the effects varies with position and protease in spite of high sequence homology. Mechanistic investigation of papain proteolysis reveals that the thioamide effects derive from reductions in both affinity (K_M) and turnover number (k_{cat}). Computational modeling allows these effects to be understood based on disruption of key enzyme-substrate hydrogen bonds, providing a model for future rational use of thioamides to confer cysteine protease resistance.

Thioamide substitution, a single atom O-to-S modification of the peptide backbone, has been shown to reduce the rate of proteolysis when incorporated at or near the scissile bond.[1] For example, our laboratory described the introduction of thioamides into glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), two therapeutically relevant peptide substrates of dipeptidyl peptidase 4 (DPP-4).[2] A single thioamide near the scissile bond provided up to 750-fold enhancement of peptide stability compared to the all-amide peptide without significantly altering cellular activity, and a thioamide modified GLP-1 was active in rats. A recent report by Chatterjee and coworkers demonstrated that thioamide incorporation in macrocyclic integrin antagonists can increase their serum half-life by 3-fold while maintaining, or even increasing, potency toward cancer cell lines.[3] Studies such as these show that thioamides can be highly useful for the stabilization of peptides toward proteolytic events for *in vivo* applications (although it should be noted that renal clearance and other factors impact *in vivo* half-life).[4] However, despite numerous investigations of proteases using thioamide peptides, few systematic studies have been performed to study the positional effects of thioamide substitution on proteolysis.[1h, 1j]

Taylor M. Barrett, Xing Chen, John J. Ferrie, E. James Peters
Department of Chemistry
University of Pennsylvania
231 South 34th Street, Philadelphia, PA 19104, USA
E-mail: ejpetersson@sas.upenn.edu
Chunxiao Liu
Department of Applied Chemistry
China Agricultural University
Yuanmingyuan West Road 2, Beijing 100193, China

Supporting information (SI) for this article is given via a link at end of the document.((Please delete this text if not appropriat

Our laboratory has previously developed a thioamide-based fluorescent protease sensor system which we envisioned using to better understand the effects of thioamides on proteolysis.[5] This system, based on our extensive studies of thioamides as fluorescence quenchers,[6] involved placing a thioamide and a fluorophore on opposite sides of the scissile bond, so that a fluorescence turn-on would be observed once proteolysis occurs. To study the positional effects of thioamides on cysteine proteases, we generated a peptide substrate labeled with a fluorophore, 7-methoxycoumarin-4-yl-alanine (μ), at both the C-terminus and N-terminus with the thioamide placed at the position to be tested (Fig. 1). With this design, no matter where the thioamide is located, quenching by one of the fluorophores will be relieved when the peptide is cleaved, so that proteolysis can be monitored by a fluorescence change. In the case of the P1 thioamide, successful proteolysis should lead to dequenching of both fluorophores, since the thionoacid resulting from initial thioamide hydrolysis would undergo further reactions with water to form the carboxylic acid.[1a, 1c] Figure 1 shows the fluorescence turn-on scheme for the P2 thioamide construct (others are shown in SI Fig. S2). Our sensor design provides a general method for testing thioamide positional effects using real-time fluorescence to monitor cleavage kinetics, a significant improvement over earlier designs that either required chromatographic analysis or only provided information on non-prime positions.[1c, 1e, 1h, 7]

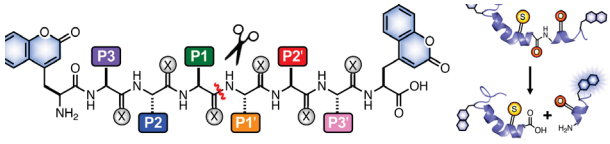


Figure 1. Design of Thioamide Positional Scanning Probes. Peptides contain 7-methoxycoumarinylalanine (μ) residues at the termini, and amide (X=O) or thioamide (X=S) residues between. The red line represents the cleavage site. Amino acid residues are denoted as P3 to P3' from the N-terminus to the C-terminus. P2 peptide fluorescence turn-on shown corresponds to Fig. 2 inset.

For an initial demonstration of our method, we investigated cysteine proteases in order to compare our results to previous, more limited studies of thioamide effects on these enzymes and show the value of an exploration of many positions and proteases, enabled by our sensor design.[1d, 8] Cysteine proteases are primarily localized in the cytosol or lysosome, and mediate general functions such as catabolism of intracellular proteins as well as specialized functions like selective activation of extracellular protein degradation[9]. The papain family cysteine proteases, which include parasite proteases (cruzain and falcipains), plant enzymes (bromelain and papain), and human cysteine cathepsins (Cts B, C, F, H, K, L, O, S, V, W, X), are the

most abundant cysteine proteases. They play important roles in many biological and pathological events.[10] Thus, the ability to manipulate cysteine proteases by inhibiting them, making peptides resistant to them, or designing sensors for them can have valuable applications in biology and medicine.

We studied papain as well as Cts B, K, S, and V (31-59% homologous, SI Fig. S15). For these experiments, a series of peptides, μLLK/AAAμ and its thioamide analogs, were synthesized using slightly modified solid phase methods, employing thioacylbenzotriazole monomers to introduce the thioamides as previously described.[11] The sequence was designed to be recognized by all five cysteine proteases and cleaved at the position indicated by "/", based on our own previous study and comprehensive sequence/activity studies of these proteases by Ellman and coworkers.[5, 12] We generated variants where the thioamide was scanned from the P3 position to the P3' position, using the standard protease nomenclature as depicted in Figure 1. For example, the P1 substrate has the sequence μLLK^{S}AAAμ, where the position of the thioamide is indicated by a superscript "S."

In our initial steady state protease assay, each peptide substrate was incubated at 7.5 μM in the presence or absence of a protease in a concentration determined to lead to complete cleavage of the Oxo control substrate (μLLKAAAμ) within 30 min. While the thioamide substrates were designed to have a turn-on of fluorescence, we found that the oxoamide substrate was also conveniently fluorogenic, presumably due to relief of quenching through a homo-FRET (Förster resonance energy transfer) mechanism.[13] Thus, for all substrates, fluorescence intensity was monitored over time to determine cleavage kinetics (see Fig. 2 Inset and Fig. S3-S7 in SI for primary data). Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) was used to identify the Oxo peptide fragments generated by proteolysis and confirm that significant cleavage took place only at the designed site, between Lys and Ala (Fig. S8, SI). In the absence of protease, no significant change in fluorescence was observed, and no degradation was observed by MALDI MS. By monitoring the increase in fluorescence over time until proteolysis was complete, we could convert the fluorescence data to product formation rates, as detailed in the SI. The normalized proteolysis rate data shown in Figure 2 allow one to easily visually compare the effects of thioamidation at each position for each protease. While all proteases were affected by at least one thioamide position, the magnitude of the effects at each position were greatly variable according to the protease. For a more quantitative comparison, the normalized initial rates of each peptide with each protease are reported in Table 1.

We found that thioamides which were far away from the scissile bond, at the P3 and P3' positions, had little effect on the peptide half-life. Thioamides near the scissile bond conferred different levels of resistance to proteolysis. Similar to previous results from Cho *et al.* and Asbóth *et al.*, we found that papain proteolysis was dramatically slowed by a P1' or P2 thioamide, while only slightly affected by the P1 thioamide.[1d, 8] However, this result is clearly not applicable to all cysteine proteases. While all five proteases were slowed to a similar extent by P1' thioamidation, the effect of P2 thioamidation was variable. Cts B

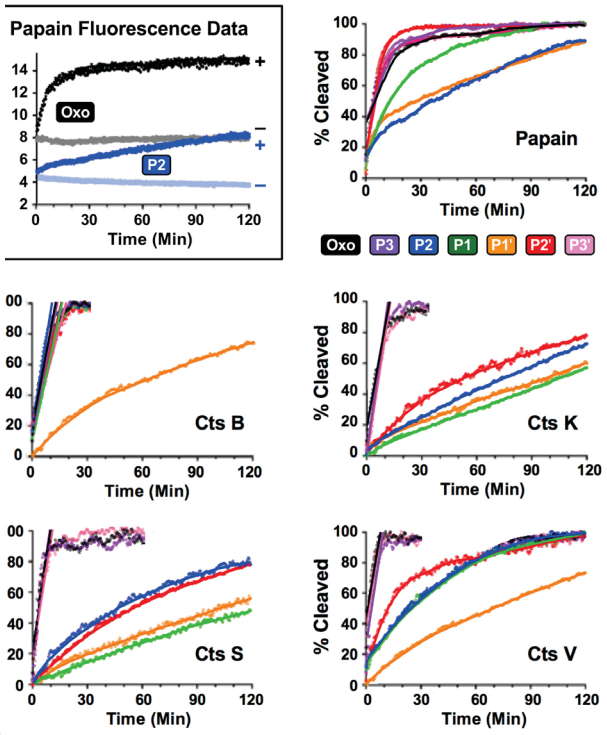


Figure 2. Thioamide Positional Effects on Proteolysis Rates. Top Left Inset: Primary fluorescence data for Oxo control peptide and P2 thioamide peptide (both defined in Fig. 1) in the presence (+) and absence (–) of papain. Top Right: Background-corrected, normalized fluorescence traces for papain proteolysis of Oxo control peptide and P3, P2, P1, P1', P2', and P3' thioamide peptides (defined in Fig. 1), colored black, purple, blue, green, orange, red, and pink as shown. Bottom: Fluorescence traces for proteolysis by cathepsins (Cts) B, K, S, and V. All traces are the average of three trials. Experimental details and primary fluorescence traces for each experiment are given in SI.

was not affected by the P2 thioamide and was only affected by the P1' thioamide. The activities of Cts K, S, and V were reduced by thioamides at all positions except P3 and P3'. Cts K and S were significantly retarded by P1 thioamides, in contrast to our papain data and the previously published results.[1d, 1e]

	0.10 ± 0.01[b]	1.18 ± 0.07	0.16 ± 0.01	17 ± 0.01
	1.18 ± 0.09	0.70 ± 0.04	1.03 ± 0.11	13 ± 0.11

To probe the mechanism of thioamidation effects, we chose to study papain in more depth because significant, but site-selective effects were observed, and because more structural data are available for substrates of papain than Cts B, K, S, or V. We studied papain proteolysis at various concentrations of the Oxo, P2, and P1' peptides to determine Michaelis-Menten kinetic parameters. We found that the catalytic efficiency (k_{cat}/K_M) for papain cleavage of the thioamide peptides was about 10-fold lower than that of the Oxo peptide (Fig. 3). For both the P2 and P1' peptides, the reduction in k_{cat}/K_M resulted from a combination of a 2-fold change in K_M and a 5-fold change in k_{cat}. Thus, it seems that the P2 or P1' thioamide has a minor effect on binding to papain, and a more significant effect on adopting a productive cleavage conformation. While previous studies have attributed thioamide protease resistance to the higher rotational barrier of the thioamide (thioamide: 22 kcal/mol, amide: 14 kcal/mol), these effects can also be explained merely by accounting for altered hydrogen bonding around the thioamide (see below).[1i, 14]

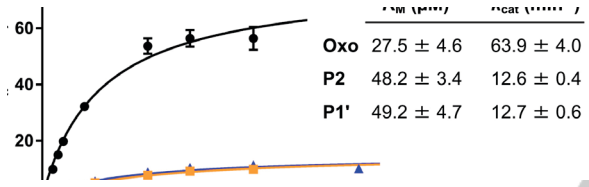


Figure 3. Michaelis-Menten Analysis of Papain Activity on Thioamide Peptides. Various concentrations of Oxo, P2, or P1' peptide were incubated in the presence of 1.2 μM papain. Primary fluorescence data are shown in SI Fig. S10.

The k_{cat} effect contrasts with our finding that thioamides at the P2 or the P1 position of DPP-4 substrates primarily affect binding, and thus the thioamide peptides do not serve as competitive inhibitors of DPP-4.[2] In this case, binding without proteolysis could make the P2 and P1' thioamide peptides competitive inhibitors of other papain substrates, so we assessed their inhibition of the proteolysis of a commercial chromogenic papain substrate, L-pyroglutamyl-L-phenylalanyl-L-leucine-*p*-nitroanilide (PFLNA). However, 50 μM concentrations of the thioamide peptides did not significantly inhibit PFLNA proteolysis (Fig. S11 and Table S7, SI), indicating that they are not potent competitive inhibitors, as expected based on their K_Ms. Solubility problems at higher concentrations prevented us from determining whether they functioned as weak (K_I >100 μM) competitive inhibitors.

In order to better understand the thioamide positional effects, we conducted peptide docking simulations and examined existing X-ray structures of papain with covalent peptide inhibitors bound. While several papain structures are available in the Protein Data Bank (PDB) with inhibitors mimicking P3, P2, and P1 interactions (PDB IDs 1PAD, 1CVZ, 1PPP), only one structure (PDB ID 1BP4) exists with an inhibitor mimicking P1', P2', and P3' interactions.[15] We found that the geometry obtained with Rosetta FlexPepDock was consistent with the X-ray structures and shed further light on the P1' interaction.[16] The docked structure is shown in Figure 4, highlighting interactions of carbonyl groups with papain which may be disrupted by thioamide substitution. Additional papain structures are shown in SI (Figs. S12-14, S17-S18, and S23-S25).

The P1' carbonyl (Fig. 4, orange) interacts with His_{159}, which serves as the catalytic base, deprotonating Cys_{25} for nucleophilic attack on the P1 amide bond. Thioamidation could weaken stabilizing interactions with the protonated form of His_{159}, leading to the decrease in k_{cat} for the P1 thioamide peptide. The P2 amide (Fig. 4, blue) makes both hydrogen bond donor (with the Asp_{158} backbone carbonyl) and acceptor (with the Gly_{66} amide N-H) interactions. Thioamidation could weaken the acceptor interaction and strengthen the donor interaction, repositioning the P2 site closer to Asp_{158}, altering active site geometry and again leading to a decrease in k_{cat}. The mild, two-fold effect on proteolysis of the P1 thioamide (Fig. 4, green) can be attributed to disruption of hydrogen bonding interactions with the "oxyanion hole" Gln_{19} sidechain and Cys_{25} backbone amides, repositioning the scissile bond prior to nucleophilic attack and/or destabilizing the tetrahedral intermediate.[1e] The variability of the positioning of the P1 carbonyl and Gln_{19} sidechain in the model (Fig. 4) and papain X-ray structures (SI Figs. S17-S18, S23-S25) implies that this interaction is flexible and helps to explain its small effect. The P3, P2', and P3' positions (Fig. 4 and Figs. S12-S14, purple, red, pink, respectively) are relatively solvent exposed, with few interactions, consistent with their lack of effect on proteolysis.

Analysis of Cts B, K, S, and V structures (Figs. S19-S22, SI) with inhibitors bound to the non-prime side demonstrates that the P2 amide makes hydrogen bonds comparable to those in the docked structure (Fig. 4) in all cases. However, the carbonyl hydrogen bond acceptor in Cts B is found in a flexible Gly_{197}-Gly_{198} sequence, which could tolerate the thioamide perturbation. In the other proteases, the corresponding carbonyl is found in a rigid Val/Leu-Asp/Asn sequence, such as the Val_{157}-Asp_{158} site in papain (Fig. 4). This may explain why Cts B is not affected by P2 thioamidation. In this way, prediction of thioamide effects on proteolysis must account not only for altered binding interactions, but also for dynamics that allow the enzyme to compensate for the thioamide perturbation.

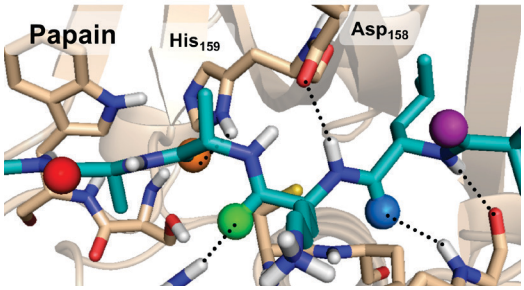


Figure 4. Interactions of Substrate Carbonyls in Papain Active Site. Docked structure of LLKAAA peptide shows carbonyl interactions of the P3 (purple), P2 (blue), P1 (green), P1' (orange), and P2' (red) residues. Key hydrogen bonds shown as dashed lines. Details of computational modelling are given in SI.

In summary, we studied the positional effects of thioamide substitution on proteolysis by the homologous cysteine proteases papain, Cts B, Cts K, Cts S and Cts V. For all five proteases, P1' thioamidation significantly affected proteolysis, indicating that this may be a modification that can be used to stabilize injectable peptides toward cleavage by papain family cysteine proteases in a manner similar to our GLP-1 and GIP DPP-4 stabilization.[2] In all cases except for Cts B (lowest homology to other Cts enzymes, SI Fig. S15), other thioamide positions also had a 10-fold or greater effect, allowing for flexibility in one's placement of a thioamide for stabilization if one found that a P1' thioamide compromised bioactivity of the peptide. No protease tested was affected by a thioamide at the P3 or P3' position. This finding is useful because it allows one to place a thioamide at either of these positions to make turn-on sensors for papain-family cysteine proteases without fear of disrupting protease activity. Thus, our study exemplifies how a systematic approach enabled by our sensor peptide design establishes guidelines for employing thioamides in both peptide medicinal chemistry and fluorescent biosensor applications. In addition, these substrates can be used to rigorously analyze Michaelis-Menten kinetics to provide mechanistic insight when coupled with structural modelling, as we have shown for papain. The generality of our sensor peptide strategy will allow us to perform similar studies on other protease classes to provide broad sets of such rules for peptide chemists.

Experimental Section

Details of thioamide precursor synthesis, peptide synthesis and purification, fluorescence assays, MS analysis, computational modelling, and analysis of existing crystal structures is given in Supporting Information (SI).

Acknowledgements ((optional))

This work was supported by the University of Pennsylvania and the National Science Foundation (NSF CHE-1708759 to E.J.P.). Instruments supported by the NSF and National Institutes of Health include: MALDI MS (NSF MRI-0820996), LCMS and HRMS (NIH RR-023444), stopped flow fluorometer (NSF CHE-1337449), and NMR (NIH RR-022442). T.M.B. thanks the NIH for funding through the Chemistry Biology Interface Training Program (T32 GM071399). J.J.F. thanks the NSF for funding through the NSF Graduate Research Fellowship Program (DGE-1321851).

Keywords: Thioamide • Protease • Papain • Cathepsin • Fluorescence

[1] aP. A. Bartlett, K. L. Spear, N. E. Jacobsen, *Biochemistry* **1982**, *21*, 1608-1611; bR. E. Beattie, D. T. Elmore, C. H. Williams, D. S. Guthrie, *Biochem. J.* **1987**, *245*, 285-288; cM. D. Bond, B. Holmquist, B. L. Vallee, *J. Inorg. Biochem.* **1986**, *28*, 97-105; dK. Cho, *Anal. Biochem.* **1987**, *164*, 248-253; eK. L. Foje, R. P. Hanzlik, *Biochim. Biophys. Acta* **1994**, *1201*, 447-453; fL. Maziak, G. Lajoie, B. Belleau, *J. Am. Chem. Soc.* **1986**, *108*, 182-183; gJ. McELROY, D. J. GUTHRIE, N. M. HOOPER, C. H. WILLIAMS, Portland Press Limited, **1998**; hM. Schutkowski, M. Jakob, G. Landgraf, I. Born, K. Neubert, G. Fischer, *FEBS J.* **1997**, *245*, 381-385; iM. Schutkowski, K. Neubert, G. Fischer, *FEBS J.* **1994**, *221*, 455-461; jS. Yao, R. Zutshi, J. Chmielewski, *Bioorganic Med. Chem. Lett.* **1998**, *8*, 699-704.

[2] X. Chen, E. G. Mietlicki-Baase, T. M. Barrett, L. E. McGrath, K. Koch-Laskowski, J. J. Ferrie, M. R. Hayes, E. J. Petersson, *J. Am. Chem. Soc.* **2017**, *139*, 16688-16695.

[3] H. Verma, B. Khatri, S. Chakraborti, J. Chatterjee, *Chem. Sci.* **2018**, *9*, 2443-2451.

[4] B. Manandhar, J. M. Ahn, *J. Med. Chem.* **2015**, *58*, 1020-1037.

[5] J. M. Goldberg, X. Chen, N. Meinhardt, D. C. Greenbaum, E. J. Petersson, *J. Am. Chem. Soc.* **2014**, *136*, 2086-2093.

[6] aJ. M. Goldberg, S. Batjargal, B. S. Chen, E. J. Petersson, *J. Am. Chem. Soc.* **2013**, *135*, 18651-18658; bE. J. Petersson, J. M. Goldberg, R. F. Wissner, *Pys. Chem. Chem. Phys.* **2014**, *16*, 6827-6837.

[7] B. Asboth, L. Polgar, *Biochemistry* **1983**, *22*, 117-122.

[8] B. Asboth, E. Stokum, I. U. Khan, L. Polgar, *Biochemistry* **1985**, *24*, 606-609.

[9] S. Zucker, J. Cao, W.-T. Chen, *Oncogene* **2000**, *19*, 6642.

[10] aF. Lecaille, J. Kaleta, D. Brömme, *Chem. Rev.* **2002**, *102*, 4459-4488; bD. Bromme, J. Kaleta, *Curr. Pharm. Des.* **2002**, *8*, 1639-1658; cM. Sajid, J. H. McKerrow, *Mol. Biochem. Parasit.* **2002**, *120*, 1-21; dP. J. Rosenthal, *Int. J. Parasitol.* **2004**, *34*, 1489-1499.

[11] aD. Szantai-Kis, C. Walters, T. Barrett, E. Hoang, E. Petersson, *Synlett* **2017**, *28*, 1789-1794; bM. A. Shalaby, C. W. Grote, H. Rapoport, *J. Org. Chem.* **1996**, *61*, 9045-9048.

[12] Y. Choe, F. Leonetti, D. C. Greenbaum, F. Lecaille, M. Bogyo, D. Brömme, J. A. Ellman, C. S. Craik, *J. Biol. Chem.* **2006**, *281*, 12824-12832.

[13] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3 ed., Springer US, **2006**.

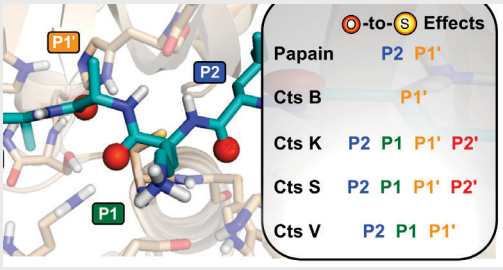
[14] K. B. Wiberg, D. J. Rush, *J. Am. Chem. Soc.* **2001**, *123*, 2038-2046.

[15] aJ. M. LaLonde, B. Zhao, W. W. Smith, C. A. Janson, R. L. DesJarlais, T. A. Tomaszek, T. J. Carr, S. K. Thompson, H.-J. Oh, D. S. Yamashita, D. F. Veber, S. S. Abdel-Meguid, *J. Med. Chem.* **1998**, *41*, 4567-4576; bJ. Drenth, K. H. Kalk, H. M. Swen, *Biochemistry* **1976**, *15*, 3731-3738; cH. Tsuge, T. Nishimura, Y. Tada, T. Asao, D. Turk, V. Turk, N. Katunuma, *Biochem. Biophys. Res. Commun.* **1999**, *266*, 411-416; dM. J. Kim, D. Yamamoto, K. Matsumoto, M. Inoue, T. Ishida, H. Mizuno, S. Sumiya, K. Kitamura, *Biochem. J.* **1992**, *287*, 797.

[16] B. Raveh, N. London, O. Schueler-Furman, *Proteins* **2010**, *78*, 2029-2040.

Entry for the Table of Contents (Please choose one layout)

COMMUNICATION



Xing Chen,[a] John J. Ferrie,[a] and E. James Petersson[a]

Page No. – Page No.

Fluorescent Probes for Studying Thioamide Positional Effects on Proteolysis Reveal Insight into Resistance to Cysteine Proteases