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Inactivation of α_1-Proteinase Inhibitor as a Broad Screen for Detecting Proteolytic Activities in Unknown Samples

Daniel Nelson,* Jan Potempa,† and James Travis*,[1]

*Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602; and
†Department of Microbiology and Immunology, Jagiellonian University, Krakow, Poland

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The need for a quick, simple screening method for the detection of general proteolytic activity prompted us to determine whether cleavage within the reactive site loop region (RSL) of α_1-proteinase inhibitor (α_1-PI), a well-characterized member of the serpin family known to be susceptible to proteolytic inactivation, can be utilized for this purpose. Inactivation of α_1-PI in the RSL region can be measured by loss of residual inhibitory capacity of α_1-PI against its target proteinase. While we originally utilized this assay to detect a new proteinase from culture supernatants of *Porphyromonas gingivalis,* the feasibility of extending this assay to scan for proteolytic activity from other systems was also assessed. As an example, we found that the serine proteinase from *Staphylococcus aureus* (SSP) had virtually the same catalytic efficiency in inactivating α_1-PI in our assay as it did in the hydrolysis of the synthetic substrate Z-Phe-Leu-Glu–pNA (k_{cat}/K_m value of 2×10^4 M^{-1} s^{-1} vs 2.6×10^4 M^{-1} s^{-1}, respectively). Additionally, in both assays activity could be readily detected in less than a 1 h incubation at SSP concentrations in the picomolar range. This assay is unique in that proteinases which hydrolyze peptide bonds within the RSL of α_1-PI can readily be detected as measured by loss of α_1-PI inhibitory activity.

Various assay systems are currently in use to follow the activities of proteolytic enzymes. Most of these methods involve the release of a covalently bound reporter group of a chromogenic, fluorogenic, isotopic, or immunological variety, with each having advantages and disadvantages in terms of sensitivity, specificity, cost, assay time, equipment needed, background levels, and utilization of hazardous or radioactive reagents (1–8). Although such techniques can efficiently quantitate enzyme activity, they often only mimic physiological substrates in their native state. This is particularly true for peptide–pNA[2] substrates because they represent only the truncated primary structure (P_1 subsites only) and are not generally useful in detecting metalloproteinases and aspartyl proteinases (recognition of P_1' subsites) or highly evolved specific proteinases that may require secondary or tertiary structure binding sites for catalysis to proceed. Currently, a study of the action of proteinases on native or denatured proteins requires an incubation period followed by separation of degradation products, usually by electrophoresis or chromatography. Furthermore, some form of quantitation such as scanning densitometry or mass spectroscopy must be applied to obtain useful kinetic data. Thus, these techniques have been mostly avoided and, consequently, many novel proteinases may remain unidentified.

One area where the native structure may be important for hydrolytic events is in the family of plasma serine proteinase inhibitors (serpins), a group of proteins which is characterized by an exposed reactive site loop (RSL) domain which acts as a "bait" region for a specific endogenous target proteinase (9). Significantly, this unique structure also leaves residues exposed for proteolytic attack by nontarget proteinases, either of host or nonhost origin, wherein cleavage leads to a conformational change of the RSL and subsequent inactivation of the inhibitory capacity (10).

Magnotti (11) has recently shown that the quantity of one proteinase can be determined indirectly by measuring a decrease in activity of a secondary proteinase

[1] To whom correspondence should be addressed. Fax: (706) 542-3719.

[2] Abbreviations used: α_1-PI, human α-1 proteinase inhibitor; pNA, *para*-nitroanilide; HNE, human neutrophil elastase; SSP, staphylococcal V-8 serine proteinase; RSL, reactive site loop; MMP, matrix metalloproteinase; Z, benzyloxycarbonyl.

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in the presence of small molecular weight inhibitors competing for both proteinases. We wished to determine if a similar approach could be employed using a serpin and a known target proteinase to detect additional unknown proteinases in a given sample. For our experiments, we utilized α_1-proteinase inhibitor (α_1-PI), a well-characterized member of the serpin family, together with one of its target proteinases, α-chymotrypsin. Indeed, we noted that an uncharacterized factor from culture supernatants of *Porphyromonas gingivalis* was able to cleave α_1-PI despite the fact that none of the known proteinases from this organism possessed this ability. We, therefore, were able to use this assay as a means of following this new activity during its purification (manuscript in preparation). In the present study, we wanted to determine if this α_1-PI inactivation assay would be suitable for general use as a screening tool to detect unknown proteinases from various sources which may have physiological implications. To this end, we chose to use the staphylococcal serine proteinase (SSP) from strain V-8 of *Staphylococcus aureus* as the model nontarget proteinase because it has the ability to hydrolyze synthetic peptide–pNA substrates (12) as well as within the RSL structure of native α_1-PI (13). These properties allowed us to compare both the kinetic efficiency and sensitivity of an α_1-PI inactivation assay against the more traditional peptide–pNA assay.

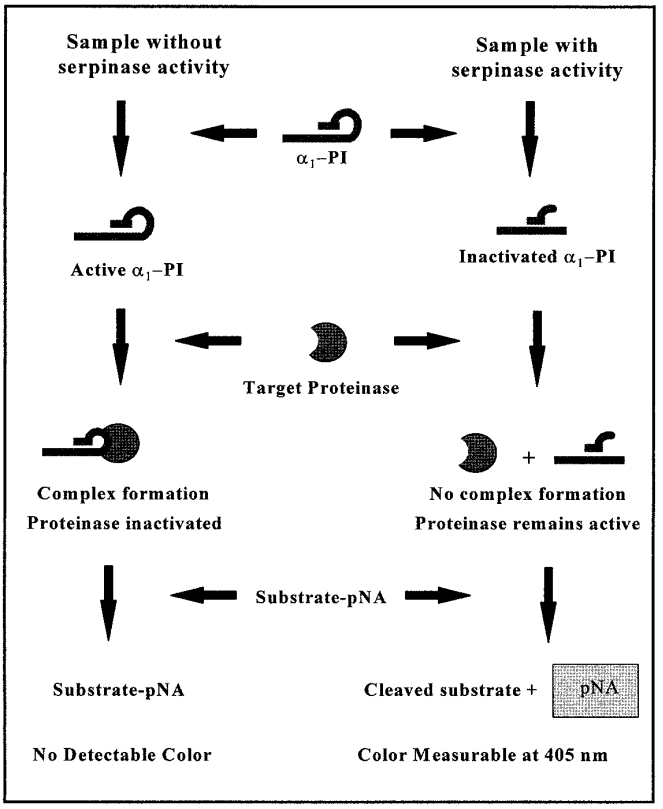

FIG. 1. Schematic diagram of the α_1-PI inactivation assay. The "target proteinase" for α_1-PI can be HNE, α-chymotrypsin, or trypsin and the corresponding "peptide–pNA" would be *N*-Suc-Ala-Ala-Pro-Val–pNA, *N*-Suc-Ala-Ala-Pro-Phe–pNA, or *N*-benzoyl-Arg–pNA, respectively. For the experiments carried out in this paper, α-chymotrypsin and *N*-Suc-Ala-Ala-Pro-Phe–pNA were utilized.

MATERIALS AND METHODS

All buffers, reagents, and peptide substrates were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated, and were of analytical grade. SSP (14), human α_1-PI (15), HNE (16), and human α-chymotrypsin (17) were purified in this laboratory as previously described.

Theory of Assay

α_1-PI is a 1:1 molar irreversible inhibitor of human neutrophil elastase (HNE) *in vivo*, as well as α-chymotrypsin and trypsin *in vitro* (9). All of the above target proteinases utilize standard chromogenic substrates to detect activity at 405 nm (*N*-Suc-Ala-Ala-Pro-Val–pNA for HNE, *N*-Suc-Ala-Ala-Pro-Phe–pNA for α-chymotrypsin, and *N*-benzoyl-Arg–pNA for trypsin). If equimolar amounts of α_1-PI and target proteinase are allowed to complex, there will be no detectable change in absorbance at 405 nm upon addition of the chromogenic substrate for the target proteinase. Preincubation of α_1-PI with a "serpinase" activity before addition of the target proteinase causes a reduction of functional α_1-PI, allowing any uncomplexed target proteinase to act upon it's chromogenic substrate. The activity of target proteinase on chromogenic substrate would then be directly proportional to serpinase activity of the nontarget proteinase on α_1-PI (Fig. 1).

α_1-Proteinase Inhibitor Inactivation Assay

All assays were carried out in buffer A (100 mM Tris, 0.02% NaN_2, pH 7.4). Human α-chymotrypsin was titrated against α_1-PI to determine the volume of active stock necessary to completely inhibit 0.15 nmol of α_1-PI (data not shown). α_1-PI (3 nmol) was mixed with various concentrations of SSP in a final volume of 100 μl of buffer A (to give a final physiological concentration of 30 μM for α_1-PI) and incubated at 37°C. At desired time intervals, 5 μl of reaction mixture (0.15 nmol of α_1-PI) was removed and mixed with exactly 0.15 nmol of α-chymotrypsin from titration in a final volume of 990 μl of buffer A. Using these assay conditions, we could generate 20 different time points from a single digestion if necessary. After allowing the α_1-PI/α-chymotrypsin mixture to complex for 1 min at room temperature, 10 μl of 50 mM *N*-Suc-Ala-Ala-Pro-Phe–pNA in dimethyl sulfoxide was added to the mixture and

allowed to react for 4 min at 25°C before the reaction was stopped by the addition of 50μl glacial acetic acid. Endpoint absorbance at 405 nm was read in a Molecular Devices SpectraMax Plus spectrophotometer and the percentage α_1-PI remaining was calculated from controls (no α_1-PI, 0.15 nM α-chymotrypsin) versus (0.15 nM α_1-PI, 0.15 nM α-chymotrypsin) in the 4-min reaction with Suc-Ala-Ala-Pro-Phe–pNA.

Studies on P. gingivalis Proteinases and Vesicles

P. gingivalis strain ATCC 33277 was grown under standard conditions according to ATCC. Preparation of vesicles, HRGP, RGP-2, and KGP was performed as previously described (18, 19) and resuspended in buffer A. The assay was carried out utilizing 30 μM α_1-PI and aliquots were assayed at 0, 30, 60, 90, 120, 180, and 240 min. To one sample of vesicles, 5 μM of the cysteine proteinase inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) was added prior to incubation with α_1-PI.

Determination of k_{cat}/K_m

The inactivation of α_1-PI under pseudo-first-order conditions can be followed by an exponential decay progress curve according to an integral form of the Michaelis–Menten:

$$S_t = S_0 \cdot \exp(-k_{cat}/K_m \cdot E \cdot t) \quad [1]$$

where S_t is the substrate (α_1-PI) concentration at time t, S_0 is the initial substrate concentration, and E is the SSP concentration (20). At the half-life of such a progress curve, S_t/S_0 is 1/2 and Eq. [1] can then be further rearranged to yield

$$k_{cat}/K_m = \frac{\ln 2}{E \cdot t_{1/2}} \quad [2]$$

where $t_{1/2}$ is the time in seconds required to inactivate one half of the α_1-PI. In all assays, α_1-PI was kept constant at the physiological plasma concentration of 30 μM. By varying the enzyme concentration of SSP, we were able to generate inactivation curves for α_1-PI after being normalized to our 100 and 0% controls. Statistical analysis of the data using Prism v2.01 by GraphPad Software yielded substrate depletion curves that fit a one-phase exponential decay regression indicating we were under Michaelis–Menten conditions. The apparent value of k_{cat}/K_m was determined from the $t_{1/2}$ of the exponential decay curves fitted to the data for each SSP concentration according to Eq. [2].

Kinetics for the Peptide–pNA Assay

Kinetic readings were carried out under continuous measurement at 405 nm in a Molecular Devices SpectraMax Plus spectrophotometer at 25°C. SSP (79 nM) in a final volume of 200 μl of buffer A was placed in triplicate rows on a 96-well microtiter plate and allowed to equilibrate to 25°C. Various concentrations of Z-Phe-Leu-Glu–pNA ranging from 2 mM to 7 μM in a final volume of 50 μl were added to the microtiter plate simultaneously through a multichannel pipet and read kinetically for 10 min. The data set was limited to the linear portion of the plot to represent the initial velocity for each substrate concentration. This measurement, in mOD/min, was transformed with the extinction coefficient for pNA of 9650 M^{-1} cm^{-1} to yield an initial velocity in terms of moles/second after correction for the path length of the machine. The initial velocity, along with substrate concentration in moles, was entered into the shareware program Hyperbolic Regression Analysis of Enzyme Kinetic Data v. 1.02a which yielded V_{max} and K_m values from which other kinetic parameters were calculated.

Sensitivity of the Two Assays

The detection limits for both assays were assessed by determining the lowest amount of proteinase that was detectable within 1 h by making serial dilutions of SSP and assaying under standard conditions. For the α_1-PI inactivation assay, incubation was with SSP and 30 μM α_1-PI followed by the 4-min development assay, whereas the 1-h incubation for the peptide pNA assay utilized SSP and 10 mM Z-Phe-Leu-Glu–pNA, with the 405-nm endpoint absorbance taken. Positive detection was determined to be either 10% inactivation of α_1-PI compared to the 100% control in the α_1-PI assay or 10% absorbance of released pNA compared to a 100% control representing the endpoint of 10 mM free pNA. The 10% figure was determined arbitrarily because it represented the smallest relative cleavage that we could easily replicate which was clearly above background limits.

RESULTS

Inactivation of native human α_1-PI was assessed in an indirect assay by measuring residual inhibitory activity of this serpin on human α-chymotrypsin, as measured with the α-chymotrypsin substrate, *N*-Suc-Ala-Ala-Pro-Phe–pNA. While our original experiments were carried out with the endogenous target proteinase for α_1-PI, HNE, and its peptide substrate, *N*-Suc-Ala-Ala-Pro-Val–pNA (data not shown), we chose to carry out the reported experiments on α-chymotrypsin due to greater accessibility and lower cost of obtaining highly purified enzyme relative to HNE. This was justified by

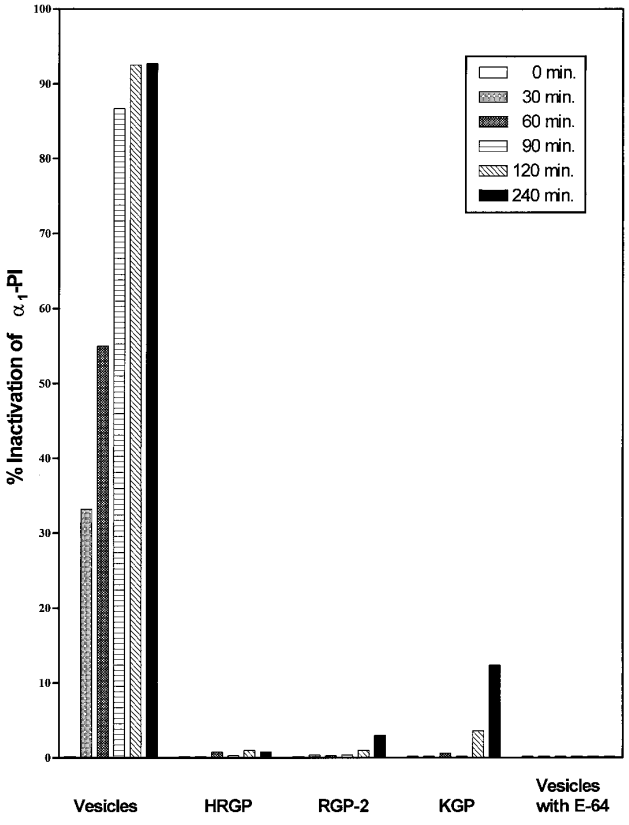

FIG. 2. Time course inactivation of α_1-PI by vesicles and individual proteinases from *P. gingivalis*. 30 μM α_1-PI was incubated with 5 μM purified proteinase or fresh vesicles for indicated periods of time before assaying. Vesicles pretreated with 5 μM E-64 showed no activity even when the incubation was extended to 72 h.

the fact that α_1-PI *in vitro* is a 1:1 molar inhibitor of HNE, trypsin, and α-chymotrypsin.

The α_1-PI inactivation assay was originally employed to study culture supernatants of *P. gingivalis*, which is characterized by numerous cysteine proteinases (KGP, RGP-2, and HRGP) which are membrane bound, secreted, or present on vesicles released from this organism (for review see Ref. 21). Using this assay we noted an activity, not attributable to the known proteinases, that appeared to inactivate α_1-PI and was inhibited by E-64 (Fig. 2). Further purification and characterization of this new activity revealed that it was unable to cleave azocasein, collagen, or any peptide–pNA, including those with residues mimicking the primary sequence of the RSL (manuscript in preparation). For this reason, we had to utilize the α_1-PI inactivation assay exclusively for studying this new activity. We then sought to determine how comparable the α_1-PI inactivation assay was with the more standard peptide–pNA assays. To this end, we used SSP because it has activity against α_1-PI and a peptide–pNA, plus it removes any bias in the data toward the new *P. gingivalis* proteinase.

Various dilutions of SSP were incubated with α_1-PI (30 μM) for 2 h with aliquots removed for assay at regular intervals. The serpinase activity of SSP on α_1-PI was halted by the addition of an equimolar amount of previously active-site titrated α-chymotrypsin. When complexed with this target proteinase, α_1-PI undergoes a conformational change and no longer is sensitive to proteolysis by nontarget proteinases (SSP). Although we allowed 1 min at room temperature for formation of the complex, we noted in separate experiments that mixing equimolar amounts of α_1-PI and α-chymotrypsin followed 1 s later by *N*-Suc-Ala-Ala-Pro-Phe–pNA produced no detectable activity at 405 nm, indicating that complex formation was nearly instantaneous and that our time points were accurate.

Development of the assay was carried out by detecting activity of any uncomplexed α-chymotrypsin against *N*-Suc-Ala-Ala-Pro-Phe–pNA in a final concentration of 0.2 mM for 4 min. This concentration and reaction time was arbitrarily decided upon because even in the 100% control (no α_1-PI, 0.15 nM α-chymotrypsin), the 4-min assay yielded a kinetic curve that was still linear, indicating substrate depletion had not taken place before the reaction was stopped by the addition of glacial acetic acid. The inactivation of α_1-PI generated one-phase exponential substrate decay curves with an R^2 value greater than 0.99 for all enzyme to substrate ratio's ranging from 1:500 to 1:10,000 (Fig. 3). Half-life values were fit into Eq. [2] to generate apparent k_{cat}/K_m values for each curve. The average k_{cat}/K_m value of 2×10^4 M^{-1} s^{-1} for the α_1-PI inactivation assay was comparable to the value of 2.6×10^4 M^{-1} s^{-1} found with the peptide–pNA assay. To show that the constant k_{cat}/K_m value was linear over all enzyme concentrations, the observed rate constant, k_{obs}, was plotted vs SSP concentration which gave a linear relationship (Fig. 4). Comparison of k_{cat}/K_mbetween the two assays indicates similar catalytic efficiency. Furthermore, both assays had the ability to detect proteolytic activity of SSP in the subnanomolar range in a 1-h assay under standard conditions (Table 1).

DISCUSSION

HNE is the target enzyme which irreversibly binds with α_1-PI in a 1:1 molar ratio, after which the complex is removed from the circulation either by phagocytic cells or by receptors in the liver (22, 23). Proteolytic inactivation of α_1-PI causes a localized imbalance of the proteinase/inhibitor ratio favoring active HNE and leading toward unregulated proteolysis and tissue damage. Because of the large number of matrix metalloproteinases (MMPs) that inactivate α_1-PI, it is believed that free HNE would aid in the breakdown of basement membrane proteins in the tissue remodeling

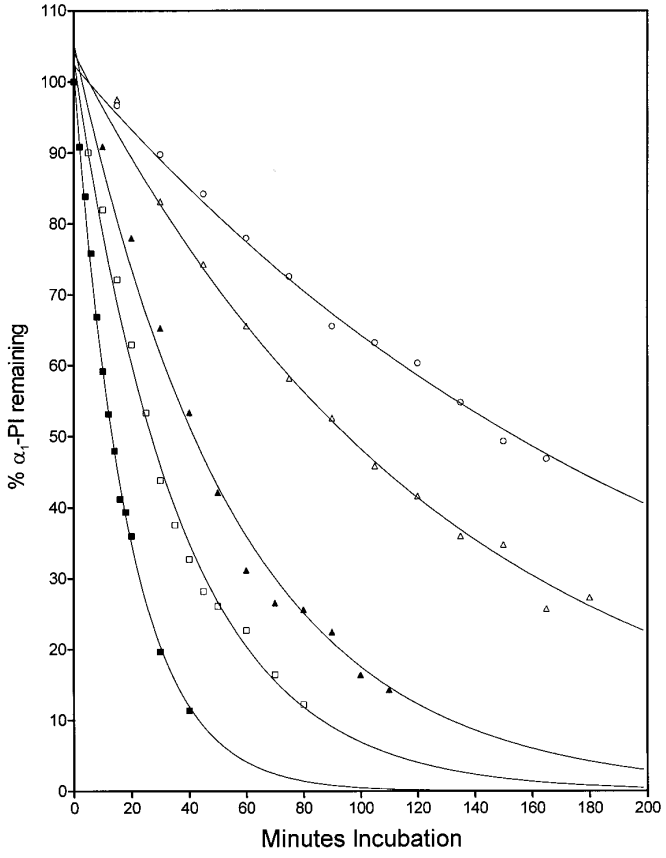

FIG. 3. Inactivation of α_1-PI by SSP vs time. α_1-PI concentration was kept constant at 30μM and SSP molar concentration varied as indicated: 1:500 (■); 1:1000 (□); 1:2500 (▲); 1:5000 (△); and 1:10000 (○).

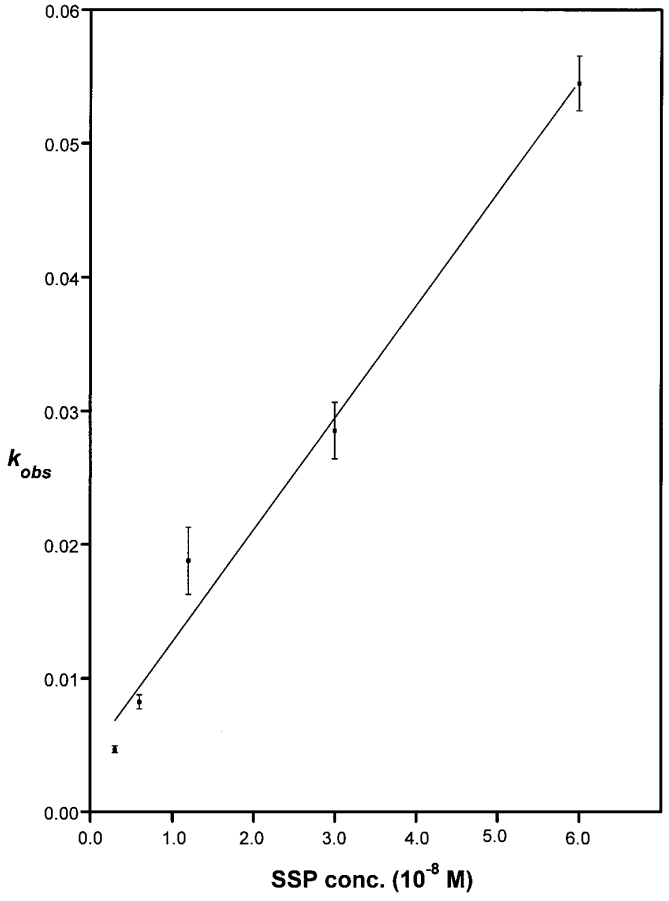

FIG. 4. k_{obs} vs SSP concentration. The observed rate constant, k_{obs}, calculated from the exponential decay curves indicates that k_{cat}/K_m values are linear over a range of SSP concentrations.

process (20, 24, 25). While this may be beneficial in the case of MMPs, the proteinase/inhibitor imbalance is more often associated with diseases such as pulmonary emphysema (26), periodontal disease (27), Alzheimer's disease (28, 29), cystic fibrosis (30, 31), adult respiratory distress syndrome (32), and sepsis (33, 34). In infected tissues, there is a large influx of neutrophils with subsequent release of HNE. Thus, any bacterial proteinase that can inactivate α_1-PI should be considered as a virulence factor since unregulated proteolysis by HNE would elaborate additional nutritive supplies for an invading microorganism. Indeed, the growing list of nontarget proteinases from a variety of nonhost sources would seem to confirm this hypothesis (Table 2). It is important to note that several of the proteinases listed in Table 2 cleave the RSL of α_1-PI at residues differing from their published specificity, suggesting that other structure factors may play a more important role in recognition than primary residue specificity. Recent elucidation of the crystal structure indicates that the RSL region of serpins is an exposed β-pleated sheet (35, 36) which may be converted to a random coil structure during binding of specific or non-specific proteinases. This may explain why several of the MMPs, which have activity against denatured substrates such as gelatin, can cleave α_1-PI at seemingly odd residues. This being the case, the α_1-PI inactivation assay as we have described it may be used as a screening tool for general nonspecific proteolytic activities.

Additionally, due to its physiological relevance, there is growing evidence that inactivation of α_1-PI may occur not only by these nonspecific actions but also by highly evolved proteinases acting on secondary and

TABLE 1

Comparison of the α_1-PI Inactivation Assay and the Standard Chromogenic Assay for SSP

	α_1-PI inactivation assay	Z-Phe-Leu-Glu-pNA assay
k_{cat}/K_m	2.0×10^4 M^{-1} s^{-1}	2.6×10^4 M^{-1} s^{-1}
Detection limit	<1 nM	<500 pM

TABLE 2

The RSL Sequence for α_1-PI and Known Cleavage Sites by Both Endogenous and Nonhost Proteinases

G A M F L E A I P **M S** I P P E
1 2 3 4 5 6

	Ref.
1. *Crotalus adamanteus* proteinase II	(39)
Stromelysin − 3 (MMP-11)	(38)
2. *S. aureus* metalloproteinase	(13)
S. aureus cysteine proteinase	(13)
Tissue collagenase (MMP-1)	(20, 40)
Neutrophil collagenase (MMP-8)	(24, 25, 41)
Neutrophil gelatinase (MMP-9)	(25, 41)
3. *S. aureus* V-8 proteinse (SSP)	(13)
House dust mite allergen (Der P1)	(42)
Cathepsin L	(43)
4. House dust mite allergen (Der P1)	(42)
5. Tissue collagenase (MMP-1)	(40)
P. aeruginosa elastase	(44)
Matrylisin (MMP-7)	(45)
Stromelysin (MMP-3)	(20, 45, 46)
Neutrophil collagenase (MMP-8)	(24, 41)
Neutrophil gelatinase (MMP-9)	(41)
6. Papain	(47)
S. marcescens metalloproteinase	(48)
Cathepsin L	(43)
Ragweed pollen trypsin-like peptidase	(49)
Seaprose	(50)

Note. The Met-Ser residues indicated in bold form the bond attacked by HNE which results in subsequent inhibition of HNE.

tertiary binding sites in order to alter the HNE/α_1-PI balance. As described earlier, the *P. gingivalis* proteinase discovered using this assay apparently displays a distinct activity for the α_1-PI RSL. A clearer example of narrow substrate specificity by a well-characterized proteinase involves stromelysin 3, a member of the MMP family which has a characteristic catalytic domain, a zinc-binding consensus domain and a "hemopexin-like" domain (37). However, unlike the other MMPs, stromelysin 3 does not, apparently, have the ability to degrade any of the extracellular matrix components such as fibronectin, vitronectin, laminin, gelatin, elastin, or types I and IV collagen (38). Indeed, the only known substrate for stromelysin 3, found so far, is native α_1-PI. These results, taken together, support the fact that there may be several, as yet, undetected proteinases which require tertiary binding sites on a target serpin for catalysis to proceed, and which would be detectable by this assay.

In this study, the apparent catalytic efficiency and sensitivity of an α_1-PI inactivation assay was assessed in relation to a standard proteolytic assay. One drawback of this assay as we have described it is that it does not allow calculation of exact values for V_{max} and K_m because the substrate concentration is not varied. Although it is possible to obtain these parameters, it would be a rather lengthy ordeal because each substrate concentration of α_1-PI would have to be individually titrated against the α-chymotrypsin to determine stoichiometric amounts for inhibition. While useful when working with a pure proteinase that has a known α_1-PI inactivation activity, it is impractical for general screening or purification purposes. However, the assay as we have described here does allow for an approximate measure of catalytic efficiency (k_{cat}/K_m) at physiological concentrations, and with a high degree of sensitivity. Furthermore, by changing the target proteinase for α_1-PI, the specificity of the reporting substrate changes (Phe for chymotrypsin, Arg or Lys for trypsin, and Val for HNE). This flexibility allows the α_1-PI inactivation assay to be used even under conditions in which crude samples may have multiple proteolytic activities. This is exemplified by our ability to use this assay in the presence of very strong trypsin-like proteinases of *P. gingivalis* by using α-chymotrypsin and a chymotrypsin substrate as shown in Fig. (2).

Finally, the principles of this assay can be extended to the rest of the serpin family of inhibitors by using the appropriate target proteinase in a 1:1 molar ratio with the specific chromogenic substrate for the target proteinase. For example, a serpinase activity against α-antichymotrypsin could be detected by substituting this serpin for α_1-PI and still utilizing all other reagents from the α_1-PI inactivation assay. In like manner, antithrombin III inactivation could be followed using its target enzyme, thrombin, with the thrombin substrate, phenylalanyl-pipecolyl-arginine–pNA (Phe-Pip-Arg–pNA). Since all of the serpins differ in amino acid constituents of the RSL, probing multiple serpins in this manner can be used to elucidate almost any proteolytic activity in an unknown sample. Inactivation of serpins by proteolytic cleavage, whether by nonspecific actions, sequence specificity of the RSL, or tertiary binding sites on the native serpin, provides an additional tool for the detection of novel proteolytic enzymes.

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