A Simple Immunoassay to Detect Protease Inhibitors in Microbial Fermentation Broths

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Proteases play important roles in pathologies of digestion, inflammation, blood clotting, fertilization, the complement system as well as in bacterial, viral and fungal infection, hypertension, emphysema, malignancy and metastasis[1~6], both by post translational modification of precursor proteins and by controlling protein turnover. Consequently, they represent important targets for therapeutic agents and extensive efforts have been made to develop inhibitors of proteases as drugs. In this context natural products have proved to be a rich source for the discovery of biologically active agents.

A number of methods for assay of proteases and anti proteolytic activity on proteinaceous substrates are available by measuring the rate of hydrolysis of chromogenic or fluorigenic substrates or by employing antibodies[7~10]. But no one procedure is universally suitable given the diversity and specificity of the proteases. In fact all proteolytic enzymes exhibit preference and specificity for certain bonds and the rate of hydrolysis is determined by the nature of the amino-acid in the scissile bond. Definition of the substrate is therefore very important for understanding how the inhibitor controls the mechanism of process.

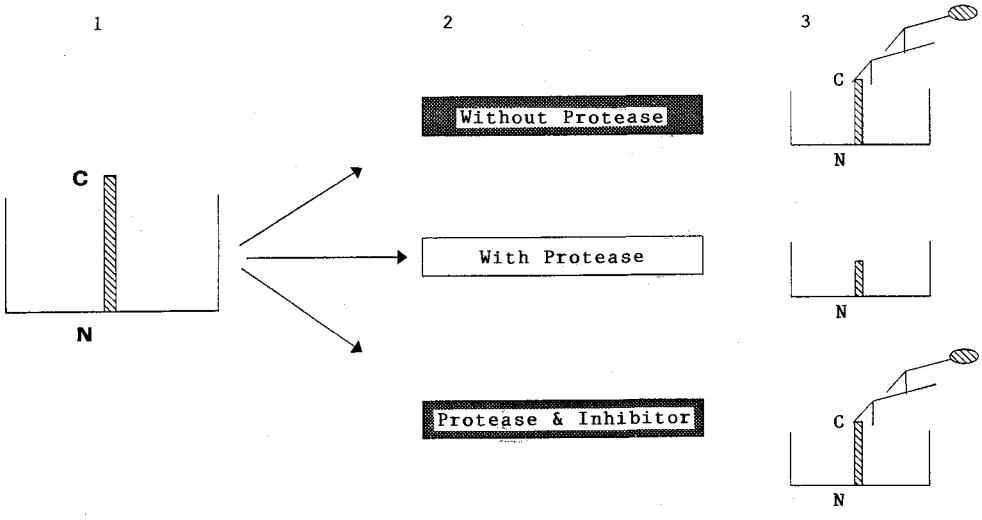
We describe a simple assay which uses a single substrate and a single detection system for proteolytic enzymes belonging to different classes. Tubulin, the microtubule subunit protein, is known to be proteolytically cleaved by several classes of proteases and constitutes an universal substrate[11~13]. Furthermore, a specific monoclonal antibody which recognises the C-terminal tyrosine residue is commercially available. These two reagents have allowed us to develop an assay, shown schematically in Fig. 1, to detect inhibitors of different classes of proteases and at the same time to provide information on the specificity and potency of the molecule.

Materials and Methods

Substrate Preparation

Bovine brain tubulin was obtained according to PEDROTTI *et al.*[14]. Briefly, whole brain was homogenized in PIPES buffer containing 0.1 mM PMSF and 40 μg/ml leupeptin, after centrifugation the supernatant was adjusted to 20% glycerol and 1 mM GTP and incubated at 37°C for 20 minutes. At the end of the incubation period the protein solution was centrifuged and the pelleted protein resuspended in MES buffer and cold dissociated for 60 minutes at 4°C. After centrifugation the supernatant was again adjusted to 1 mM GTP and a second cycle of polymerization was performed. Tubulin was purified by ion exchange chromatography using a Mono Q column (HR5/5, Pharmacia) as described in

Fig. 1. A schematic representation of the assay.



Tubulin is linked to the microtiter plate by its N-terminus to microtiter well. Protease preincubated with broth added to substrate. Uncleaved substrate recognized by C-terminus specific antibody.

1. N-terminal immobilised tubulin.
2. Incubation with buffer solution with or without protease or protease in presence of inhibitor.
3. Detection of C-terminal by YL1/2 and labelled secondary antibody.

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detail previously[14].

Covalent Linkage of the Substrate to the Support

Tubulin was then covalently linked to the microtiter well of a Covalink plate (Nunc) *via* its N-terminus by using as spacer arm BS3, bis-(sulfosuccinimide)-suberate, (Pierce, Rockford, Il). 0.150 ml of a freshly made phosphate buffered saline (PBS) solution, pH 7.3 containing 0.03 mg of BS3 were dispensed into wells. The solution was left in the wells at room temperature for 90 minutes, unreacted reagent was eliminated by washing with PBS. 0.150 ml of a PBS solution containing 5% glycine (v/v) and 0.75 μg of tubulin or peptide was then added to wells and left for 2 hours at room temperature. The solution was then removed and wells incubated for 2 hours with 0.3 ml 5% glycine PBS to saturate any remaining protein binding sites.

Determination of the Anti-proteolytic Activity

0.15 ml of a solution containing a protease and a known or an unknown inhibitor were added to the wells treated as described in the previous section and incubated for 2 hours at 37°C. Enzymes belonging to different classes were employed in this assay in a suitable buffer. Serine proteases: subtilisin (0.25 μg/ml), trypsin (0.2 μg/ml), elastase (0.5 μg/ml), chymotrypsin (2.5 μg/ml), cathepsin G (5 μg/ml) were dissolved in a 20 mM solution of Tris pH 7.4; aspartic protease: cathepsin D (5 μg/ml), pepsin (5 μg/ml), renin (5 μg/ml) and HIV-1 protease (5 μg/ml) were dissolved in a 50 mM solution of sodium acetate pH 5.5 containing 0.01% Tween 20 (Pierce); cysteine protease: papain (0.25 μg/ml) and cathepsin B (10 μg/ml) were dissolved in a 50 mM solution of sodium acetate pH 5.5 containing 5 mM cysteine. The cathepsin enzymes were from Calbiochem, renin was from Scripps and HIV-1 protease was from Bachem, all other proteases were from Sigma-Aldrich, Dorset.

Known inhibitors used were pepstatin (aspartic), alpha-1 antitrypsin (serine), leupeptin (cysteine) (Calbiochem).

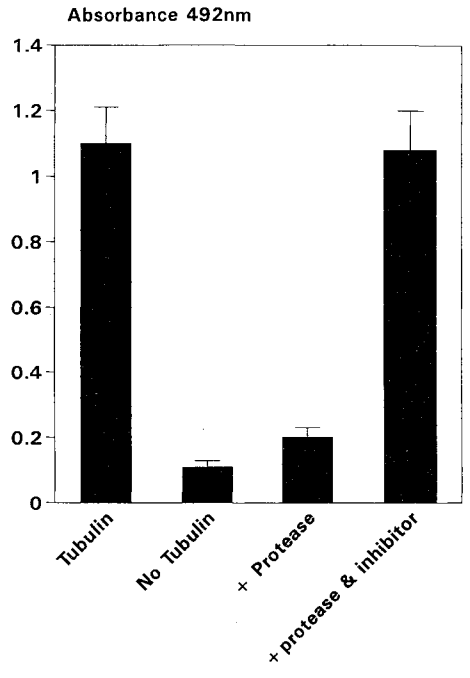
After three washes with PBS the inhibitory activity was detected as follows: 100 μl of YL 1/2 antibody solution (Amersham) diluted 1 : 1000 in PBS was added to each well and incubated 1 hour at room temperature. Wells were rinsed three times with PBS and 100 μl of anti rat-peroxidated antibody solution (Amersham) diluted 1 : 1000 in PBS was added and incubated 1 hour as before. After washing 100 μl of 1 mg/ml o-phenyldiamine (OPD) in 0.5 M citric acid pH 5.5 solution were added to each well, after 10~15 minutes the reaction stopped by addition of 100 μl of H_2SO_4 0.2 M. The colour was detected on a Titertek Multiscan MCC340-HK spectrophotometer by reading the absorbance at 492 nm. 100% inhibition represented the mean of the absorbance value obtained by the immunostaining of the uncleaved tubulin while the 0% inhibition is the absorbance value obtained by the completely digested tubulin.

Results and Discussion

Tubulin is known to be cleaved by several proteases[10~13] and is specifically recognised by antibody YL 1/2 whose epitope resides in the last 11 amino acids of the C-terminus end[15]. Pure tubulin can be covalently attached by its N-terminus and uncleaved tubulin, but not cleaved tubulin, can be detected by the C-terminus directed antibody (Fig. 1). The ability of the immobilized tubulin to act as a substrate for different proteases was assessed. Wells coated with either immobilized tubulin or buffer were challenged with either buffer, or buffer containing a protease or alternatively containing protease and its specific inhibitor. After 2 hours of incubation the buffer solution was removed and after washing with PBS the uncleaved protein was detected using the YL 1/2 antibody. As shown in Fig. 2, wells incubated in the absence of protease or in the presence of inhibitor gave a high absorbance while those in the presence of protease resulted in an absorbance close to that of uncoated wells. A dose-dependent response curve in the range of 0.25 and 3.5 μg was observed, further increase in the amount of tubulin resulted in no change in the absorbance, probably due to saturation of the well (data not shown). 0.75 μg of tubulin were therefore used for all experiments described as at this concentration the signal was ten fold higher when compared with the background.

Serine proteases (elastase, trypsin, chymotrypsin, subtilisin, cathepsin G), aspartic proteases (pepsin, cathepsin D, renin, HIV-1) and cysteine proteases (papain, cathepsin B) all efficiently cleaved the immobilised

Fig. 2. Uncoated and tubulin-coated wells were incubated either in the absence of protease or in the presence of protease or in the presence of protease-inhibitor complex.



YL1/2 antibody was used for immunodetection.

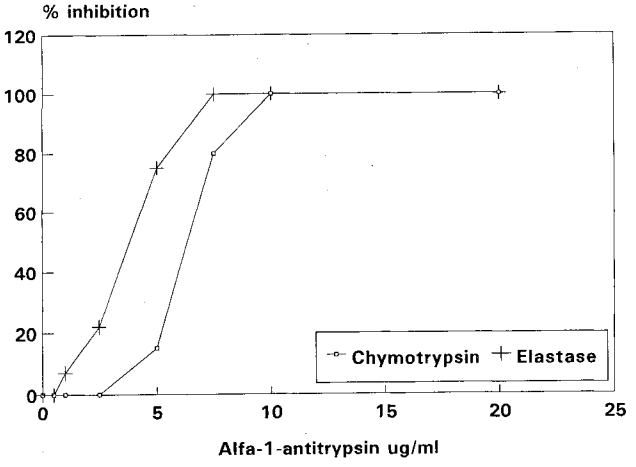
substrate and resulted in little or no absorbance development when compared with control untreated substrate. The effect of inhibitors on protease activity was also examined. Dose dependent inhibition of proteolytic activity using several different inhibitors was examined. Fig. 3 shows a typical dose dependent inhibition of alpha-1 antitrypsin on elastase and chymotrypsin.

The specific inhibition of a) pepstatin for the aspartic proteases, b) alpha-anti trypsin for the serine class proteases and c) leupeptin for thiol proteases was also determined. The concentrations which resulted in 50% inhibition of enzyme activity (IC_{50}) were calculated from dose-dependent curves (see Fig. 3). The inhibition of the different enzymes by pepstatin, leupeptin and alpha-anti trypsin was also determined (Table 1). For instance, under the test conditions, the IC_{50} of pepstatin for cathepsin D and pepsin was 20 nM (or 0.03 μg/ml) while the IC_{50} for renin was roughly an order of magnitude higher (IC_{50} = 200 nM) and for HIV-1 protease 50% inhibition was observed with 2 μM (or 3 μg/ml) pepstatin. These data are in agreement with our previous report demonstrating that pepstatin specifically inhibits the aspartic class of proteases and exhibits a higher potency against pepsin and cathepsin D when compared with HIV-1 protease[16].

Application of this assay to screen fermentation broths, however, requires a pretreatment step to eliminate any proteolytic activities which may be present in fermentation broths and consequently to avoid interference. Preliminary experiments showed that proteases could be easily eliminated by loading the broth on a mixed bed resin and elution with an organic solvent, *e.g.*, ethanol or alternatively by solvent extraction. The assay was used to screen about 6000 samples pretreated in this way. We tested about 3500 *Streptomyces* strains, 1500 *Rare actinomycetes* and 1000 *Fungi*. The percentage of samples containing protease-inhibitory activities was about 2.5%, while those exhibiting a specific activity against a single class, *i.e.* aspartic, was about 1%. We, however, failed to detect any highly selective inhibitor, *i.e.*, active against only a single enzyme. Although, using this assay we detected the presence of MAPI (microbial alkaline protease inhibitor) in one of the XAD-eluted fermentation broths[16]. Purified MAPI confirmed its ability to inhibit a number of enzymes belonging to the various classes (Table 2), in agreement with the previous data[17,18].

In searching for enzyme inhibitors, the initial screening is often done using a purified enzyme and its specific substrate. This may have several limitations the most common is non specific inhibition of the protease. Our assay is not only a high volume immunoassay, which exhibits little or no interference, but is also semiquantitative and gives information both on the specificity and potency of the inhibitor molecule. Using this assay it is possible to sort out non specific inhibitors and select for class or protease specific inhibitors. Furthermore, in this assay the detection of inhibitor requires development of colour rather than loss of signal and consequently a positive rather than a negative response (Figs. 1 and 2). A common source of interference when screening fer-

Fig. 3. Inhibition curve of alpha-1 antitrypsin against elastase and chymotrypsin.



The assay was performed as described in materials and methods section.

Table 1. Concentration of inhibitor which results in 50% inhibition of enzyme.

Protease	Pepstatin	alpha-Antitrypsin	Leupeptin
Trypsin	n.i.	1.0	n.i.
Subtilisin	n.i.	7.5	n.i.
Elastase	n.i.	1.0	n.i.
Cathepsin G	n.i.	n.i.	n.i.
Pepsin	0.03	n.i.	n.i.
Cathepsin D	0.03	n.i.	n.i.
Renin	0.30	n.i.	n.i.
HIV-1	3.0	n.i.	n.i.
Papain	n.i.	n.i.	0.05
Cathepsin B	n.i.	n.i.	2.5

Data is expressed in μg/ml, n.i. indicates the absence of inhibition. Assay conditions are reported in the materials and methods section.

Table 2. IC_{50} of MAPI determined with our assay.

Protease	MAPI
Chymotrypsin	2.5
Subtilisin	4.0
Elastase	n.i.
Cathepsin G	n.i.
Pepsin	100
Cathepsin D	n.i.
Renin	n.i.
HIV-1	10
Papain	0.08
Cathepsin B	0.6

Data is expressed in μg/ml, n.i. indicates no inhibition. Assay conditions are reported in the materials and methods section.

mentation broth is the presence of coloured extracts. This assay is not susceptible to this interference as the extracts are washed out after the reaction is terminated and prior to colour development and response evaluation.

It is also clear that any synthetic peptide containing the digestion sites of different proteases and the 11 C-terminal amino acid residues of the alpha tubulin can substitute for tubulin in the assay. We synthesized such a peptide consisting of 21 amino acid residues. Studies using this peptide showed that it could effectively substitute for tubulin in this assay (data not shown).

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