Technical Brief:

One-Step Protease Assay

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Abstract

Athena Enzyme Systems™ has developed a general protease substrate consisting of cross-linked albumin, azoalbumin, and gelatin. The substrate is susceptible to proteolysis by a wide range of enzymes including: collagenase, papain, bromelain, trypsin, chymotrypsin, proteinase K, and pronase. The assay can be run in screw-cap vials or multi-well plates for high throughput screens. Crude enzyme preparations which contain particulate matter can be assayed using a back-digestion technique for measurement of residual solid. Nucleophiles, such as thiols, in the assay buffers do not interfere. The limit of detection of enzymes is less than 100 ng for most enzymes tested. The very low labor input and "hands-on" attention are decided advantages.

Introduction

Interest in proteases has increased with the realization that they play key roles in rheumatoid arthritis (1) and cancer metastasis (2-3). Tumor progression depends on remodeling of basement membrane prior to invasion and angiogenesis. Some inhibitors of the proteases which catalyze these processes have shown good anti-tumor activity without the side effects of cytotoxic drugs.

In addition to those investigators who target proteases for research, biochemists are generally concerned with protection of their valuable proteins from unwanted degradation by contaminating proteases. These workers need to verify the presence or absence of proteases in their preparations.

Modern methods have supplanted the classical three-step assay-digestion, TCA precipitation, and detection of TCA soluble peptides by UV absorbance, the Folin-Ciocalteu reagent, or other means. Two currently popular assays are based on derivitized casein. In 1984, Twining introduced a widely applicable and sensitive assay based on fluorescein isocyanate-labelled casein (4). This assay procedure suffers from the cost of the substrate and labor intensity.

In 1992, Hatakeyama et al., reported an assay based on succinyl-casein and the TNBS reagent for detection of liberated amino groups (5). This assay can be run with the multi-well plate reader. Disadvantages include failure to detect collagenases, background due to reactive amines or thiols, and interference from particulates in the sample.

Our goal was to formulate a pipettable liquid which could be conveniently dispensed into microplates or vials. A number of cross-linking reagents and numerous sets of reaction conditions were tested before we settled on the procedure described here which employs a cross-linking agent, concentrated protein solutions and sodium benzoate.

The substrate described here is a mixture of gelatin and albumin cross-linked in the presence of sulfaniloazo-albumin at slightly acidic pH. The translucent solid is mechanically stable even at elevated temperature. Twenty-four hour backgrounds without protease are acceptably low, when the assay mixture contains 0.1 % azide. Collagenase, papain, bromelain, trypsin, chymotrypsin, proteinase K, and pronase all exhibit good activity against this protein matrix.

Materials and Methods

All chemicals were reagent grade or better. Proteins and enzymes were supplied by Sigma as follows: albumin (A2153), sulfanyloazo albumin (A2382), gelatin (G2500), collagenase C0773, papain (P4762), trypsin (T7409), chymotrypsin (C7762), proteinase K (P5568), and pronase (P0652); partially soluble enzyme preparations pancreatin (1x, 4x, and 8x) and bromelain (B22520) were used as supplied. Papaya latex (P3375) was ground to a powder before use. Assay buffers and storage solutions were prepared as previously described (6).

Vial Assays

In a typical assay, 500 µl of reaction buffer and 100 µl of enzyme solution were added to vials containing the substrate matrix, the vials sealed tightly, and incubated at 37oC. The duration of the incubation was varied from 5 min. to 24 hours. To stop the reaction, 500 µl of 0.2N NaOH was added to each vial. The absorbance at 450 nm of the aqueous phase was measured spectrophotometrically.

For enzyme samples containing particulates, such as the crude preparations studied here, a back digestion technique was employed. After reacting the crude preparation in the vial assay, the assay mixture was poured off and the solid substrate washed with water at the end of the reaction period. The extent of hydrolysis was established by digesting the remaining substrate with an excess of enzyme such as proteinase K (100 µg). The amount

of substrate digested by the crude material was determined by the difference between the no protease control (which represents the total amount of substrate) and the experimental (the residual amount of substrate). This number divided by the control value and multiplied by 100 equals the percent maximal hydrolysis.

Multi-well Plate Assays

Alternate columns of 96-well plates (Corning 25880-96) were filled with substrate. The remaining empty wells were used as working wells for absorbance measurements. A typical assay consisted of 100 µl reaction buffer containing the respective protease. After the elapsed reaction time at 37oC, 50 µl aliquots were transferred from the assay wells to the respective adjacent working wells. To each working well 50 µl of 0.1 N NaOH was added and the absorbance was read at 450 nm with a microplate reader (Molecular Devices, UV-Max).

Results and Discussion

General characteristics of the protein-gel matrix were assessed by examining matrix deterioration under different environmental conditions. The matrix was found to exhibit good thermal stability (up to 60oC, pH stability (3-12), resistance to microbial attach, and susceptibility to a wide variety of proteases including collagenase, papain, bromelain, trypsin, chymotrypsin, proteinase K, and pronase. This list encompasses a diverse range with regard to active site structure and mechanism.

Figure 1 shows time courses of the hydrolysis catalyzed by collagenase, proteinase K, papain, and chymotrypsin in the vial assay. All plots were linear for the first two hours, and within 6 h, all of the substrate was consumed. The rates are significantly higher for collagenase (25µg) and proteinase K (25µg) than for papain and chymotrypsin (50 µg of both in assay). Reactions containing lesser amounts of proteases (down to 50 ng) also showed linear rates but required up to 20 h incubation. Similar results were obtained with the microplate assay (data not shown).

Figure 2 shows a typical plot of A450 versus enzyme concentration. The assays were run overnight (16 h) with no shaking at 37oC in the vial or microplate assay. Under these conditions the reaction rates were linear. In each case, less than 30 ng of collagenase was detectable. These results demonstrated that the assay is versatile in that it can be formatted to accommodate different assay needs. By altering the incubation time, the sensitivity of the assay can be adjusted to account for different levels of protease in the samples being tested.

To assess microenvironmental effects, we examined the pH-dependencies of several enzymes. Plots for the chymotrypsin-catalyzed hydrolysis of N-acetyl-tryptophan amide and the azo-albumin gel versus pH show very similar profiles (Figure 3). These results suggest the absence of strong microenvironmental effects coming to bear as a resultof diffusional limitations at the interface between the solid substrate and the bulk solution.

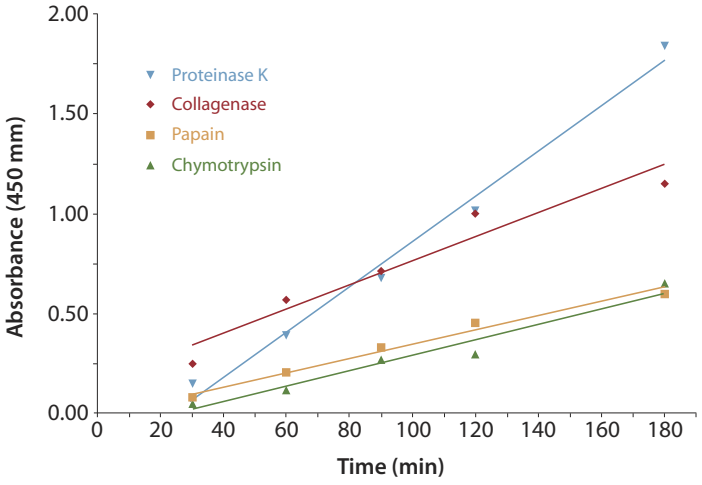

Figure 1. Time-course degradation of the matrix by papain, chymotrypsin, proteinase K, and collagenase. The reactions were incubated at 37°C and 0.2 N NaOH added to duplicate vials to stop the reaction at the indicated times. The absorbance at 450 nm was measured by pouring the reaction mixture into spectrophotometric cuvettes.

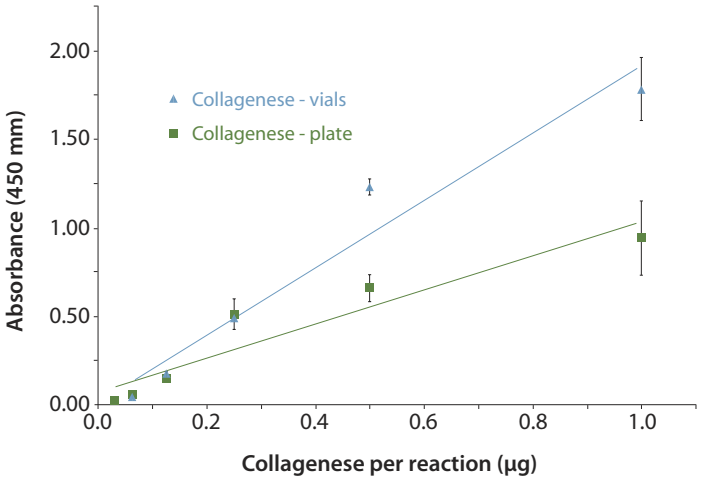

Figure 2. Rate versus enzyme concentration for collagenase for both the vial assay and the microplate assay. The limit of detection was less than 30ng.

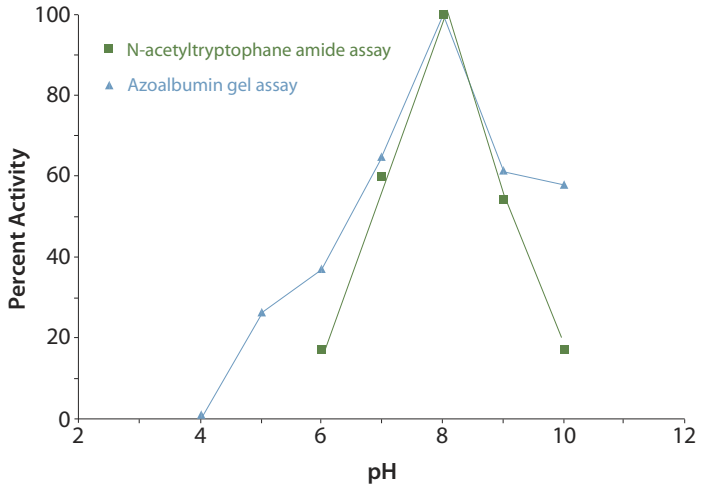

Figure 3. pH-dependences of chymotrypsin with N-acetyl-tryptophanamide and the azoalbumin substrate. The similarity of the ph-profiles would suggest the lack of any strong diffusional limitations at the interface of the solid substrate and the bulk solution.

One significant advantage of this assay system is the ability to directly test enzyme samples containing particulates. The enzyme along with suspended material was incubated with the substrate for the desired time. On completion of the reaction the vial or microplate was washed with water and the remaining substrate digested with an excess of any protease. The difference in absorbance of the control and experimental sample represents the amount of digestion. Five crude enzyme preparations which contained particulates were tested with this back digestion format. The assays were incubated for one hour at 37°C with 5 mg of crude enzyme. Following incubation, the vials were washed and the residual substrate exhaustively digested with 100 µg proteinase K. The results, shown in Table 1, were that substrate digestion using particulate containing solutions of bromelain and pancreatin was measurable by this technique. This demonstrates that protease activity in crude preparations can be quantified with this matrix assay.

Hydrolysis of Azoalbumin Gel	
Enzyme	**Percent Hydrolysis**
None	0
Bromelain	67
Pancreatin 1x	31
Pancreatin 2x	65
Pancreatin 8x	76

Table 1. The above tables shows the percent hydrolysis of azoalbumin gel, catalized by crude enzyme peparations containing particulate matter.

This assay may be useful as a high throughput screening device for detection of protease inhibitors such as matrix metalloproteinase inhibitors. To illustrate, we used the microplate format to study the inhibition of trypsin by pancreatic trypsin inhibitor. Trypsin (2 µg, 2.24 BAEE units) was mixed with different amounts of bovine trypsin inhibitor and incubated for 5 min. The residual trypsin activity was measured in the microplate assay. The results were consistent with known stoichiometry (Figure 4).

The assay described here is a rapid, low-cost, easy-to-use means of measuring protease activity. Through different formats, the assay can be applied to a rapid spot test (vial assay) for routine protease test during the preparation of biologicals, or when formatted in a multi-well microplate, applicable to high-throughput screens used to identify pharmaceutically active compounds. To devise an assay with a specific minimum cut-off would be straightforward. The reaction time and thickness of the substrate layer could be set to reflect the desired sensitivity. A microplate well exhibiting a .hit. or positive fraction would have solid substrate remaining above background as judged by direct reading or by back-digestion.

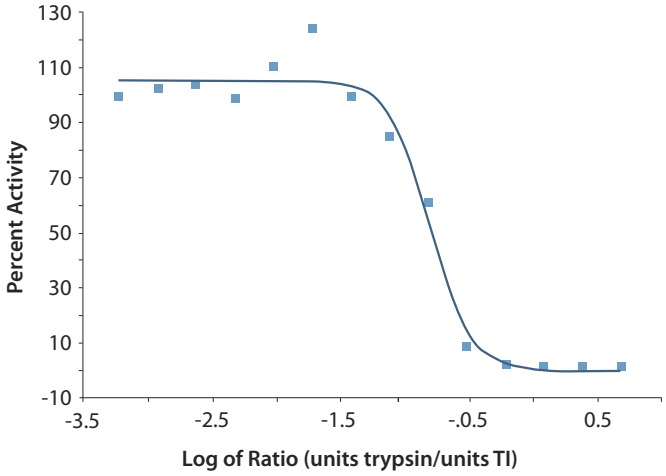

Figure 4. Dose-dependent inhibition of trypsin by bovine trypsin inhibitor. Inhibitor at various concentrations, was incubated with 2 µg (2.24 BAEE units) of trypsin for 5 min. Residual trypsin activity was measured in the microplate protease assay as described in Material and Methods for 20 h at 37°C.

References

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