

## SINGLE MICROASSAY FOR MATRIX DEGRADING ENZYMES

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### 1. ABSTRACT

Matrix degrading enzymes are implicated in several disease processes such as abdominal aortic aneurysms and emphysema, however, monitoring proteolytic activity in a single assay is not well-established. Numerous assays have been developed to measure matrix degrading enzymes, which use artificial substrates or substrates derived from natural substrate protein. We have recently developed an assay for elastolytic activity based on the detection of primary amines, using trinitrobenzene sulfonic acid (TNBSA), following the digestion of succinylated elastin. The assay is also versatile enough to allow the detection of other proteases through the use of succinylated substrate specific for given protease.

In order to improve the sensitivity and versatility of the assay we have refined the buffer conditions (PBS pH 7.2/1 mM CaCl<sub>2</sub>) to provide a 60 % increase in sensitivity to elastolytic activity and also formulated a substrate mixture containing succinylated elastin, collagen and gelatin. The combination of a substrate mixture and an optimum buffer will allow a spectrum of enzymes to be detected in a single reaction, providing a more complete picture of total proteolytic activity in a biological sample. This assay may also provide a tool to use proteolytic activity as a marker to monitor pathologic conditions involving matrix turn-over.

### 2. INTRODUCTION

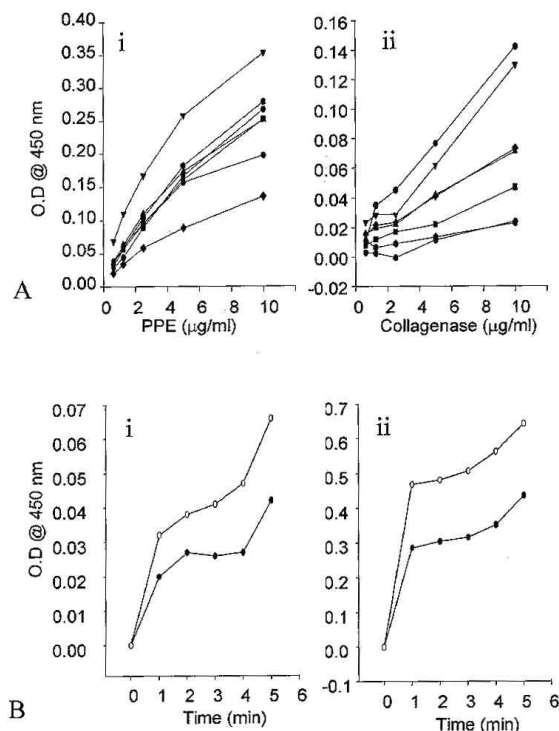
Matrix degrading proteases are implicated in many disease pathologies such as, abdominal aortic aneurysm (1.), emphysema (2.), herniation (3.), cancer (4,5.) and arthritis (6.). Many assays have been developed to measure matrix-degrading enzymes. Several assays were

developed using radiolabelled or fluorescently labeled protein substrate (7,8.), synthetic substrates (9.), zymography (4.) and ELISA (10.). However, these techniques have limitations, such as low sensitivity, are time consuming and labor intensive, are not conducive to high-throughput analysis and, in some cases, are unable to distinguish between active enzyme and zymogen.

Monitoring protease activity in a single assay is not well established, as many assays have not proved useful as diagnostic or prognostic indicators of disease. This may be because synthetic substrate assays may not represent physiologically relevant activity. Additionally, even though assays using ELISA, fluorescently labeled substrates or zymography may be very sensitive, they are not conducive to routine high throughput analysis.

In order to understand the role of matrix degradation in connective tissue disorders one must be able to detect proteolytic activity in biological samples. The use of physiological parameters as indicators of disease has been used for certain disease conditions. For example, radioimmunoassay has been used to assess pancreatitis (11.) and plasma proteins have been used, in conjunction with parameters such as tumor grade, to predict patient survival (12.)

We have previously developed a sensitive and specific independent assay for elastolytic, collagenolytic and gelatinolytic activity (13-,14) based on the detection of primary amines by 2,4,6-trinitrobenzene sulfonic acid (TNBSA), following the digestion of succinylated substrate. In this report we present an assay that incorporates a substrate mixture containing succinylated



**Figure 1.** (A) Determination of optimum digest buffer; PPE (i) and collagenase (ii) (0.01-0.0006 mg/ml) were assayed with succinylated elastin and collagen with the following buffers; 50 mM sodium borate pH 8.5, 50 mM sodium borate pH 7.2 + 130 mM NaCl + 1 mM CaCl<sub>2</sub>, PBS pH 8.5, PBS pH 7.2, PBS pH 7.2 + 1 mM CaCl<sub>2</sub>, PBS pH 7.2 + 1 mM ZnCl<sub>2</sub>, PBS pH 7.2 + 1 mM CaCl<sub>2</sub> + 1 mM ZnCl<sub>2</sub>. Reactions were incubated at 37°C for 30 min and then supplemented with 50 μl of TNBSA and incubated at room temperature for 20 min prior to determining A<sub>450nm</sub>. (B) Rate of reaction of HNE (i) and PPE (ii); reactions were carried out in triplicate in the presence of 1 μg of HNE and PPE in 50 mM sodium borate pH 8.5 and PBS pH 7.2 + 1 mM CaCl<sub>2</sub> to a total volume of 150 μl in a 96 well microtitre plate. Reactions were initiated by the addition of elastase, incubated at 37°C for 1,2,3,4 and 5 min, after which TNBSA was added and A<sub>450nm</sub> was determined after 20 min. One unit is defined as the amount of elastase that will generate 1 μM of digest product per minute at 37°C.

elastin, collagen and gelatin with PBS pH 7.2 + 1 mM CaCl<sub>2</sub> as assay buffer.

### 3. MATERIALS AND METHODS

#### 3.1. Determination of Optimum Assay Buffer

The optimum assay buffer condition was determined using 200 μg of succinylated elastin or collagen (13.) and 0.01 - 0.0006 mg/ml porcine pancreatic elastase (PPE; 270 units/mg; Calbiochem; San Diego CA) or collagenase type I (216 units/mg; Calbiochem) with 50 mM sodium borate pH 8.5, 50 mM sodium borate pH 7.2 + 130 mM NaCl + 1 mM CaCl<sub>2</sub>, PBS pH 8.5, PBS pH 7.2, PBS pH 7.2 + 1 mM CaCl<sub>2</sub>, PBS pH 7.2 + 1 mM ZnCl<sub>2</sub>, PBS pH 7.2 + 1 mM CaCl<sub>2</sub> + 1 mM ZnCl<sub>2</sub>. The reactions

were incubated at 37°C for 30 min. TNBSA (50 μl; 0.03% in 50 mM sodium borate pH 8.5) (Pierce Chemical Co, St.Louise MO) was then added and the A<sub>450nm</sub> was determined after 20 minutes at room temperature. Blank reactions contained all components except substrate.

#### 3.2. Effect of Optimum Buffer on Enzyme Rate of Reaction

The rate of reaction of human neutrophil elastase (HNE) and PPE in different assay buffers was also investigated. Reactions were carried out in triplicate in the presence of 1 μg of HNE and PPE using 50 mM sodium borate pH 8.5 and PBS pH 7.2 + 1 mM CaCl<sub>2</sub> to a total volume of 150 μl in a 96 well microtitre plate. Reactions were initiated by the addition of HNE or PPE, incubated at 37°C for 1,2,3,4 and 5 min and treated as above. One unit (U) is defined as the amount of elastase that will generate 1 μM of digest product per minute at 37°C.

#### 3.3. Combination Substrate

In addition to determining the optimum assay buffer, the use of a combination substrate containing equal amounts of succinylated elastin, collagen and gelatin substrates was investigated. The rate of reaction of 1 μg of PPE (Calbiochem) and collagenase type I (Calbiochem) individually and in combination was carried out in triplicate, using the combined substrate in 50 mM sodium borate pH 8.5 and PBS pH 7.2 + 1 mM CaCl<sub>2</sub> as described above.

### 4. RESULTS

#### 4.1. Optimum Assay Buffer

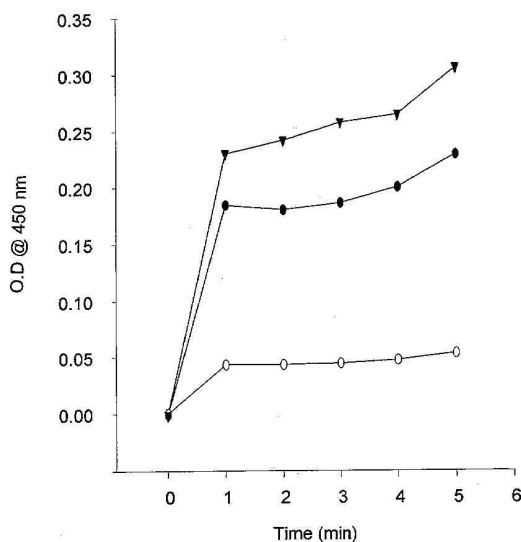
Of the buffers used (50 mM sodium borate pH 8.5, 50 mM sodium borate pH 7.2 + 130 mM NaCl + 1 mM CaCl<sub>2</sub>, PBS pH 8.5, PBS pH 7.2, PBS pH 7.2 + 1 mM CaCl<sub>2</sub>, PBS pH 7.2 + 1 mM ZnCl<sub>2</sub>, PBS pH 7.2 + 1 mM CaCl<sub>2</sub> + 1 mM ZnCl<sub>2</sub>) the optimum buffer composition was PBS pH 7.2 + 1 mM CaCl<sub>2</sub>, which increased the assay sensitivity to PPE by 54% compared to our previous assay (13). Collagenase was equally active in both PBS pH 7.2 + 1 mM CaCl<sub>2</sub> and 50 mM sodium borate pH 7.2. This suggests that using PBS pH 7.2 + 1 mM CaCl<sub>2</sub> can be used to detect both elastase and collagenase activity (Figure 1).

#### 4.2. Effect of Combination Substrate

The data indicates that the sum of the activities of PPE (0.0032 U) and collagenase (0.0007 U) alone in the presence of the combined substrate is close to the activity of a mixture of PPE and collagenase (0.004 U) in the presence of the combined substrate (Figure 2). This suggests that the combined substrate can be used to evaluate total proteolytic activity in an enzyme mixture.

### 5. DISCUSSION

The data demonstrates that using PBS pH 7.2 + 1 mM CaCl<sub>2</sub> as the digest buffer, both elastolytic and collagenolytic activity could be efficiently detected (Figure 1). The neutral pH will allow proteases with a lower optimum pH to remain active, while the presence of high salt will compensate for the reduced pH allowing proteases with higher pH requirement, such as elastase, to remain active. The combination of different substrates in a single



**Figure 2.** Combined substrate assay; assays were prepared with 200  $\mu$ g of a mixture of succinylated elastin, collagen and gelatin substrates. Reactions were carried out in triplicate in the presence of 1  $\mu$ g of PPE (●) and collagenase type I (○) (Calbiochem) individually and in combination (▼) PBS pH 7.2 + 1 mM  $\text{CaCl}_2$  to a total volume of 150  $\mu$ l in a 96 well microtitre plate. Reactions were initiated by the addition of elastase, incubated at 37°C for 1,2,3,4 and 5 min, after which TNBSA was added and  $A_{450\text{nm}}$  was determined after 20 min. One unit is defined as the amount of elastase that will generate 1  $\mu$ M of digest product per minute at 37°C.

assay will allow the detection of a spectrum of different matrix degrading enzymes.

The sum of the activities of elastase and collagenase alone is equivalent to the activity of an elastase/collagenase mixture (Figure 2) and PPE and collagenase did not mutually inhibit activity. This demonstrates that the total proteolytic activity of biological samples can be measured with this assay (Figure 2).

The concept of using a physiological parameter as a prognostic or diagnostic indicator of disease has been applied to other disease conditions. Radioimmunoassay of trypsin and fecal excretion of  $\alpha$ -2 macroglobulin have been applied to the assessment of pancreatitis and inflammatory bowel disease (11,15.). Studies of urokinase-type plasminogen activator have indicated that it could be used as a prognostic tool in conjunction with tumor grade in predicting patient survival for patients with glioma (12.). Urinary tissue factor (Utf) has been used as a marker for benign and malignant urological disease (16.). Therefore, measurement of proteolytic activity may also be applied to study and monitor disease conditions that are associated with elevated proteolytic activity and matrix degradation. Use of succinylated substrates is biologically relevant and the use of this highly specific, sensitive and physiologically relevant assay for detecting proteases in different disease

conditions may eventually lead to the development of assays that will have prognostic and diagnostic value diseases linked to changes in plasma proteolytic activity.

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