PREPARATION AND CHARACTERIZATION OF IMMOBILIZED ANTIPAIN AND CHYMOSTATIN FOR THE ISOLATION OF PROTEOLYTIC ENZYMES BY AFFINITY CHROMATOGRAPHY

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Proteolytic enzymes are nearly ubiquitous in cells as well as in extracellular fluids and structures. Our interest in the isolation of proteinases stems from studies of the production and secretion of proteinases in relation to cellular growth and development in ciliate protozoans, specifically Tetrahymena thermophila. (Straus, J. Protozool. 37, 9A, 1990). While affinity chromatography with immobilized inhibitors is a well established technique for the isolation of proteases, there has been very little use of the peptide aldehyde inhibitors (Umezawa, Meth. Enz. 45, 678-695, 1990). Peptide aldehydes such as leupeptin, calpain inhibitors I & II, antipain, and chymostatin are exceptionally strong inhibitors of proteolytic activity in cell lysates of T. thermophila (Straus, 1990, op cit.). Moreover, antipain and chymostatin each have a free carboxyl group distal to the inhibitory aldehyde moiety, which makes them suitable for linkage to an immobile amine group via a condensation reaction. Hence, we prepared antipain-agarose and chymostatin-agarose and began to characterize their chromatographic properties as part of an ongoing effort to rapidly isolate proteolytic enzymes from cellular lysates and secretions.

Antipain, chymostatin, and chymotrypsin were purchased from Sigma Chemical Company and papain was obtained from Boehringer Mannheim Biochemicals. Cellular lysates of T. thermophila (strain WH-14) were prepared from axenic cultures harvested at early stationary phase (Straus, 1990, op cit.). Diaminodipropylamine-agarose (DPA) and ethyldimethyl-aminopropyl carbodiimide (EDC) were purchased from Pierce Chemical Co. For inhibitor immobilization, 5 mg aliquots of antipain or chymostatin in 1 ml dimethylsulfoxide were mixed with 1 ml DPA in 50 mM acetate at pH 4.8, and coupled via an amide linkage by the addition 50 mg EDC (Chase, Merrill, & Williams, Proc, Natl. Acad. Sci. USA 80, 5480-5484, 1983). Coupling reactions were incubated in the dark with slow shaking for 12 h. Following coupling, the immobilized matrix was placed in a 5 ml chromatography column and washed with at least 50 volumes of 0.5 M NaCl, 10 mM HEPES, pH 7. For storage, columns were equilibrated with wash buffer containing 0.02% NaN_3 at 4° C. Control columns were prepared by incubating DPA with EDC in the absence of peptides. Prior to loading proteinases on columns, papain and chymotrypsin were dissolved in wash buffer at concentrations of 1 mg/ml and T. thermophila lysates were diluted with 0.1 volume of 10X wash buffer.

Aliquots (0.5 ml) of papain or chymotrypsin (1 mg/ml) were applied to columns (1 ml bed) and eluted with at least six volumes wash buffer. With papain and T. thermophila lysates, 1 mM dithiothreitol was included in the initial wash buffer to stabilize active site cysteines and to promote enzyme-inhibitor interactions. Subsequent elutions were performed with acidic buffer (0.05 M citrate, 0.5 M NaCl, pH 3) and chaotropic agents (6 M guanidine-HCl or 8 M urea in wash buffer). Column eluants were monitored spectrophotometrically at 280 nm to estimate

protein concentrations. Proteolytic activity in column fractions was assessed by following the digestion of azocasein spectrophotometrically at 440 nm (Straus, Parrish, & Polakoski, J. Biol. Chem. 256, 1981).

When 0.5 ml aliquots of papain (1 mg/ml) were applied to a control column, all of the protein and enzyme activity eluted in the first three ml, with 90% of the protein and activity in the 2 ml fraction. No additional protein or proteolytic activity was eluted when the column was washed with acidic buffer or 6 M guanidine-HCl (Fig. 1A). In contrast, when applied to an antipain-DPA column, only about 10% of the protein and less than 1% of the initial activity were recovered in the 2 ml fraction. Secondary washing with acidic buffer eluted less than 25% of the total protease activity and about 22% of the total protein. Further washing with guanidine-HCl eluted the bulk of protein, but no enzymatic activity was recovered in those fractions (Fig. 1B). Elution of protein and enzymatic activity by acidic buffer on subsequent trials was typically less than shown below. A small amount of protein without activity was recovered when the column was eluted with 8M urea (Fig. 1C).

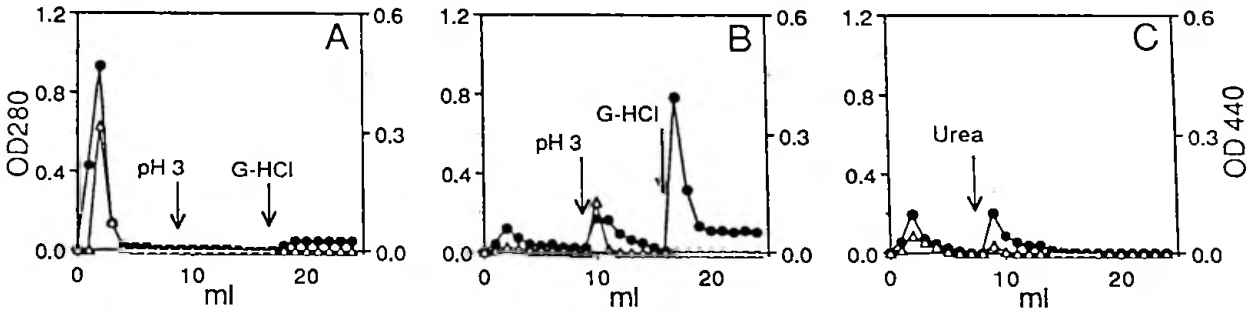

Fig. 1. Affinity chromatography of papain with antipain-DPA. OD 280 (●) indicates spectrophotometric absorption at 280 nm while OD 440 (Δ) indicates spectrophotometric absorption at 440 nm of azocasein digests, prepared by incubating 50 µl aliquots from each fraction with 0.1% azocasein in 10 mM HEPES, pH 7 for 1 h at 35° C. **A**, elution of papain from control DPA column. **B**, elution of papain from antipain-DPA column with pH 3 buffer and 6 M guanidine-HCl (G-HCl) at pH 7. **C**, elution of papain from antipain-DPA column with 8 M urea at pH 7.

Chymotrypsin behaved similarly to papain when applied to the control column, as shown in Fig 2A. There was no apparent retention of protein or enzymatic activity by the DAB matrix and further washing with acidic buffer and 6 M guanidine-HCl caused no apparent release of protein or activity. When applied to the chymostatin-DPA column, over 95% of the protein and all of the detectable enzymatic activity were retained (Fig 2B & 2C). Washing with pH 3 buffer caused a very slight release of active enzyme (less than 10% of the total applied activity) (Fig. 2B). Guanidine-HCl and urea both caused gradual elution of protein and proteolytic activity and in both cases the enzyme was spread over a large number of fractions (Fig. 2B & 2C).

When T. thermophila lysate was applied to the DPA column, there was no detectable retention

of either protein or proteolytic activity (Fig. 3A) and as with papain and chymotrypsin, further washing with acidic buffer and guanidine-HCl caused no apparent release of protein or enzyme activity. Immobilized antipain retained 65% of the total applied protein and over 90% of the proteolytic activity (Fig. 3B). No activity or protein was eluted by acid buffer. The bulk of applied protein was eluted by 6 M guanidine-HCl, but no activity was detected in these fractions (Fig. 3B). Similar results were obtained when cell lysates were applied to chymostatin-DPA (not shown). Over 50% of lysate protein and over 80% of lysate proteolytic activity were retained by chymostatin-DPA (Fig. 3C). Application of 6 M urea caused a very slight and gradual release of protein and enzyme activity. Urea caused a slight release of protein without activity from antipain-DPA (not shown).

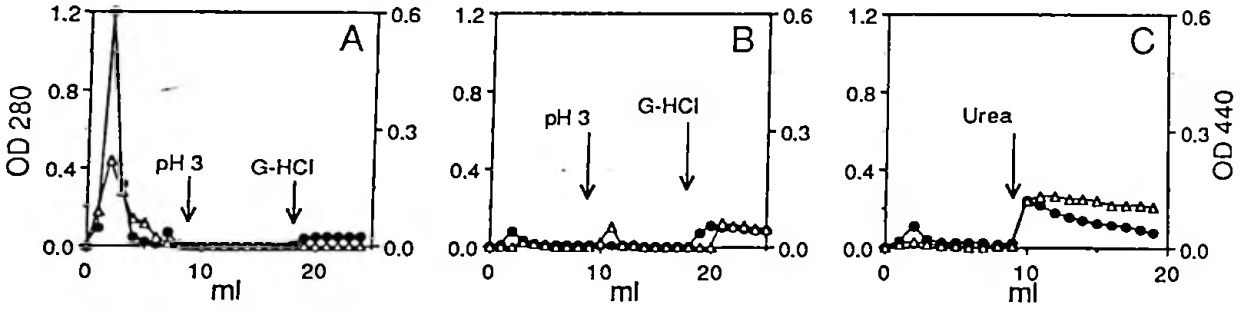

Fig. 2. Affinity chromatography of chymotrypsin with chymostatin-DPA. OD 280 (●) and OD 440 (Δ) are as described in Fig. 1. **A**, elution of chymotrypsin from control DPA column. **B**, elution of chymotrypsin from chymostatin-DPA column with pH 3 buffer and 6 M guanidine-HCl (G-HCl) at pH 7. **C**, elution of chymotrypsin from chymostatin-DPA column with 8 M urea at pH 7.

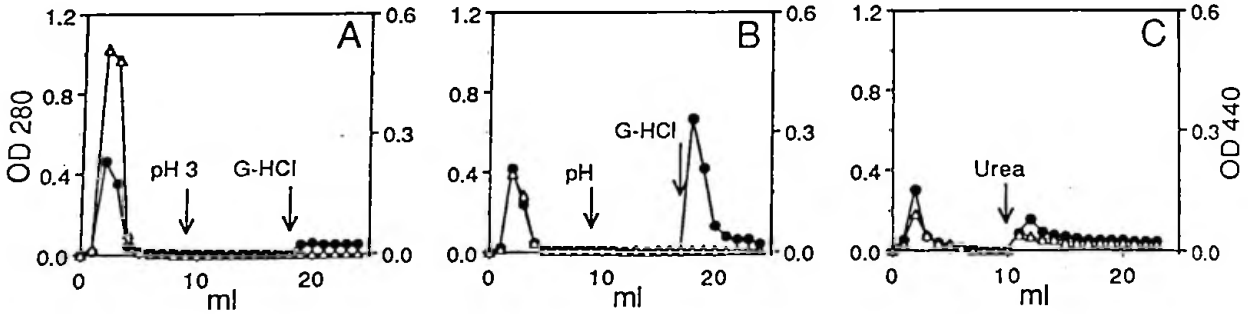

Fig. 3. Affinity chromatography of T. thermophila lysate proteases with antipain-DPA and chymostatin-DPA. OD 280 (●) and OD 440 (Δ) are as described in Fig. 1. A 1.0 ml aliquot of cell lysate was applied to columns 3A and 3C and a 2 ml aliquot was applied to column 3B. The lysate protein concentration was estimated to be 0.6 mg/ml based on absorbance at 280 nm against a bovine serum albumin standard. **A**, elution of lysate proteases from control DPA column. **B**, elution of lysate proteases from antipain-DPA column with pH 3 buffer and 6 M guanidine-HCl (G-HCl) at pH 7. **C**, elution of lysate proteases from chymostatin-DPA column with 8 M urea at pH 7.

The above results indicate that both antipain and chymostatin were successfully coupled to DPA and that both affinity matrices retained proteolytic enzymes. Attempts to recover bound protein and proteolytic activity by elution at pH 3 met with very limited success. A small proportion of bound papain and cell lysate protease were recovered when the antipain-DPA column was eluted with acidic buffer (Figs. 1B & 3B) while no protein or activity was recovered from the chymostatin-DPA column under similar circumstances (Fig. 2B). Attempts to remove bound enzymes by denaturation with chaotropic agents also met with limited success. A substantial amount of protein, but no enzymatic activity was recovered from the antipain-DPA column when washed with 6 M guanidine-HCl (Figs. 1B & 3B). Active chymotrypsin was recovered from the chymostatin-DPA column when eluted with 6 M guanidine-HCl (Fig. 2B) or 8 M urea (Fig. 2C), but release of the enzyme was slow and spread over many fractions. Urea had similar, but less dramatic effects on the elution of protein and enzyme from antipain-DPA (Fig. 1C). While the immobilized inhibitors were very effective at retaining proteolytic enzymes, acidic and chaotropic conditions were relatively ineffective in eluting active enzyme from the affinity matrices. Accordingly, more effective elution conditions will be needed to if these immobile matrices are to be used for enzyme purification. Other eluants to be tested will include active site ligands (soluble inhibitors or substrates) to compete with the immobilized ligands for enzyme.

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