# AFFINITY CHROMATOGRAPHY OF THERMOLYSIN AND OF NEUTRAL PROTEASES FROM B. SUBTILIS

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SUMMARY

Affinity chromatographic systems are described for the purification of neutral metalloendopeptidases on columns of acetyl-D-phenylalanine or succinyl-D-leucine covalently linked to Sepharose by spacers of various lengths. The neutral proteases of B. subtilis are separated in a single chromatographic procedure from all other proteins of the culture filtrates and subfractionated into two active species. An analogous chromatographic system is effective in the purification of thermolysin of B. thermoproteolyticus.

The neutral metalloendopeptidases comprise a group of microbial proteases having similar enzymatic specificities and modes of action. These enzymes catalyze the hydrolysis of peptide bonds to which the amino groups are donated by hydrophobic amino acid residues. All neutral metalloendopeptidases are optimally active at neutral pH, and all are inhibited by metal-chelating agents but not by covalent inhibitors of serine proteases or of sulfhydryl enzymes. This group of enzymes includes thermolysin from Bacillus thermoproteolyticus, "neutral proteases" from Bacillus subtilis and Bacillus megaterium and analogous enzymes from other microorganisms (1). The present report describes systems for the purification of thermolysin and of neutral proteases from B. subtilis by methods of affinity chromatography (2). These methods provide advantages over a system using immobilized substrate which has been developed independently for a neutral protease from Cl. histolyticum (3).

## MATERIALS AND METHODS

Crystalline thermolysin (from Bacillus thermoproteolyticus, Rokko) was purchased from Daiwa Kasei K. K., Osaka, Japan. A crude enzyme mixture from

Abbreviations: T, triethylenetetramine; S, succinic acid; E, ethylenediamine; HEPES, hydroxyethylpiperazine-N'-2-ethane sulfonic acid; FAGLA, furylacryloylglycyl-L-leucinamide.

the culture medium of Bacillus subtilis NRRL B3411 (Lot No. SG-2539) was obtained from the Monsanto Co. through the courtesy of Dr. J. Feder.

Adsorbents containing acetyl-D-phenylalanine were prepared by procedures similar to those described by Cuatrecasas (4) and by Uren (5). Spacers of triethylenetetramine (T), succinic acid (S) or ethylenediamine (E) were first attached in tandem to a matrix of Sepharose 4B, and then covalently coupled to the ligands.

Agarose-T-S-T-acetyl-D-phenylalanine (Adsorbent 1). Sepharose 4B (200 ml of settled gel) was suspended in an equal volume of water and activated with 4 g of CNBr at 20°. The suspension was maintained at pH 11 by the addition of 12 N NaOH. The activated agarose was washed rapidly with cold water and the packed gel was stirred overnight with an equal volume of cold 2 M triethylene-tetramine (T) at pH 9.0. Excess amine was removed by repeated washings with water. A suspension of the derivative (Agarose-T) was diluted with an equal volume of 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) (pH 7.5) and finely powdered succinic anhydride (20 g) was added over a period of 2 hr at 20°. The pH was maintained at 7.5 by the addition of 10 N NaOH. The washed gel (Agarose-T-S) was suspended in an equal volume of 2 M triethylene-tetramine (pH 5.0). A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in water was added to a final concentration of 20 mg/ml and the mixture stirred for 20 hr at 20°. After thorough washing, the gel (Agarose-T-S-T) was treated with 5 g of chloroacetyl-D-phenylalanine (5) in 200 ml of 1 M sodium arsenate (pH 11) for 20 hr at 50°. After thorough washing, the product of this reaction (Adsorbent 1) contained 4.4 μmoles of phenylalanine/ml of settled gel.

Agarose-E-S-T-acetyl-D-phenylalanine (Adsorbent 2). This product was synthesized in the same manner as Adsorbent 1 except that triethylenetetramine was replaced by ethylenediamine in the first step. Adsorbent 2 contained 1.5 μmoles of phenylalanine/ml of settled gel.

Enzymatic activity (6) towards furylacryloylglycyl-L-leucinamide (FAGLA) was followed at 345 nm using substrate (1 mM) dissolved in 0.1 M HEPES (pH

7.2) containing 0.5% dimethylformamide. Extinction coefficients were determined with a Cary Model 16 spectrophotometer, using protein concentrations measured by differential refractometry (7). Dilute protein solutions were concentrated by ultrafiltration in an Amicon cell using a UM-20E membrane at 4°.

## RESULTS AND DISCUSSION

At pH 7 both adsorbents retained thermolysin (Fig. 1) but not α-chymotrypsin

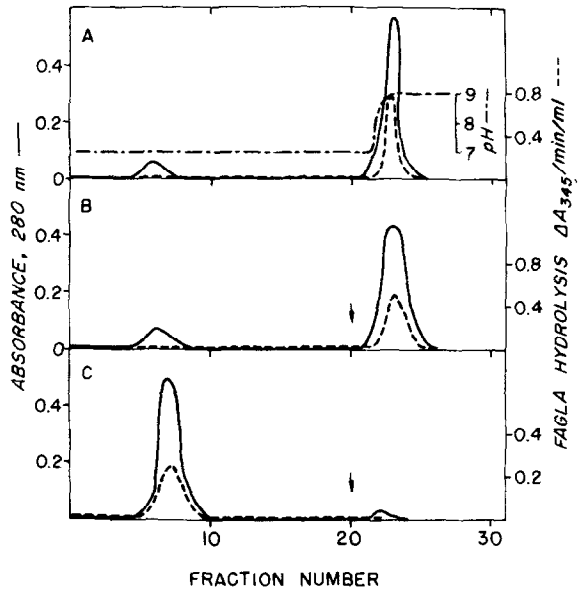


Figure 1: Affinity chromatography of thermolysin. Adsorbent 1 (8.5 ml) was first equilibrated with 10 mM HEPES (pH 7.0) containing 5 mM $CaCl_2$. The enzyme (1.5 mg) was applied in 0.5 ml of this buffer and the column was washed at 48 ml/hr for 40 min. Fractions of 1.5 ml were collected. Bound protein was then eluted with one of the following solutions: (A) 0.1 M Tris, 5 mM $CaCl_2$ (pH 9); (B) 4 M guanidinium chloride, 10 mM HEPES, 5 mM $CaCl_2$ (pH 7.0); (C) 0.1 M Tris (pH 9), 5 mM EDTA. In the last case the equilibrating buffer contained 5 mM EDTA instead of $CaCl_2$.

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or yeast protease C (8). Thermolysin was recovered from the adsorbents by elution with pH 9 buffer (Fig. 1A), or with 4 M guanidine hydrochloride (Fig. 1B). The neutral protease of B. subtilis was only weakly bound by these adsorbents at pH 7, but the affinity was greatly enhanced at pH 5 (Fig. 2). Neutral protease activity was quantitatively eluted at pH 9 and recovered in 90% yield after concentration by precipitation with 67% acetone at 4° or by ultrafiltration.

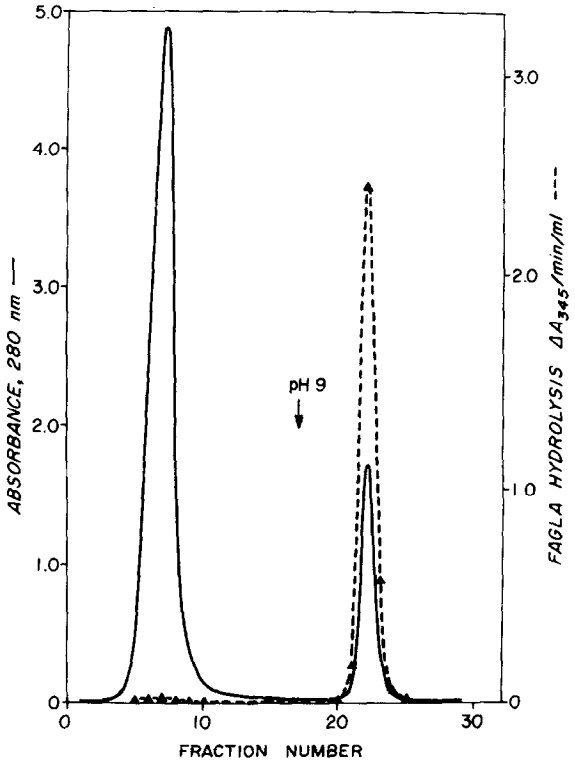


Figure 2: Purification of neutral protease by affinity chromatography. A column (2.5 x 17.5 cm) of Adsorbent 2 was equilibrated with 100 mM NaCl, 10 mM $CaCl_2$ containing 5 mM 2-(N-morpholino)ethane sulfonic acid (MES) (pH 5.0). Crude enzyme (400 mg) was applied in 4 ml of this buffer. The enzyme was eluted with 100 mM NaCl, 10 mM $CaCl_2$ containing 50 mM Tris (pH 9.0). Fractions of 15.5 ml were collected at a flow rate of 100 ml/hr.

The product appeared to be homogeneous during gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 3) and during disc gel electrophoresis at pH 8.9 in 7 M urea (9).

Similar results were obtained with two other adsorbents: one of these (Agarose-T-acetyl-D-phenylalanine) was similar to Adsorbent 1 but had a shorter spacer; the other contained succinyl-D-leucine in place of the acetyl-D-phenylalanine of Adsorbent 1. Control experiments showed that neither thermolysin

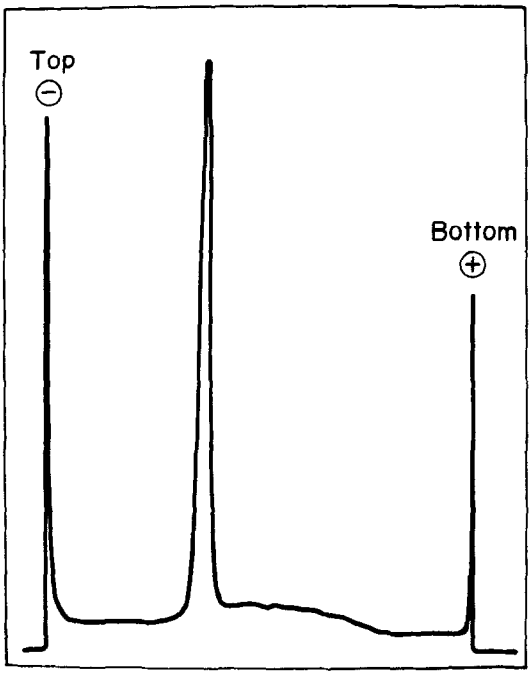


Figure 3: Gel electrophoresis of neutral protease (10 µg) from Fig. 2 in the presence of sodium dodecyl sulfate. The method of Weber and Osborn (16) was used except that thiol was omitted and a more dilute gel buffer (1.45 g $NaH_2PO_4$, 9.66 g $Na_2HPO_4 \cdot H_2O$, 2.0 g sodium dodecyl sulfate per liter) was employed. After electrophoresis (2 hr, 6 ma/gel), the gel was cut at the dye front (bottom), stained with Coomassie Blue, soaked for 2 hr in 50% methanol containing 7% $CH_3COOH$, and the excess dye removed by electrophoretic destaining. The gel was scanned at 590 nm in a Gilford (Model 2000) spectrophotometer.

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nor neutral protease was adsorbed by Sepharose derivatives which lacked the amino acid ligand.

The ligand acetyl-D-phenylalanine was specifically chosen for this work because a) peptides containing L-isomers (e.g., Cbz-Gly-L-Leu) are slowly hydrolyzed by the neutral proteases (10) and b) the enzymes can be eluted from adsorbents containing D-phenylalanine at a lower pH (where they are more stable) than from adsorbents containing D-leucine. All of the D-isomer adsorbents

appear to be stable and no change in chromatographic behavior has been observed during 50 successive uses of Adsorbent 2.

Chelating agents inhibit both enzymes (1). In the presence of 1,10-phenanthroline (1 mM) neither thermolysin nor neutral protease was adsorbed by columns of Adsorbent 1. Similarly, EDTA (5 mM) inactivated thermolysin and prevented its adsorption on this column (Fig. 1C). Protein in the breakthrough eluate regained activity when added to a substrate solution which lacked the metal-chelating agent.

Subfractionation of neutral proteases. When the neutral protease of B. subtilis was bound to Adsorbent 2 at pH 5.0 and then eluted at pH 6.3 (rather than pH 9.0 as in Fig. 2), the active enzyme emerged in two fractions differing in both chromatographic mobility and specific activity (Fig. 4). When these fractions (A and B) were separately chromatographed on the same column, each emerged as a single peak at the original elution volume. The specific activity of fraction B was three times that of fraction A (Table 1). Each species appeared homogeneous during electrophoresis as in Fig. 3. Their amino-terminal sequences were identical (Fig. 5) and their amino acid compositions were not significantly different.

No heterogeneity was evident when thermolysin was similarly adsorbed at pH 7.0 and eluted slowly at pH 8.3. The specific activity (Table 1) of the enzyme purified in this manner was greater than previously reported (11,12,13). The product appeared to be homogenous both by electrophoresis and by amino-terminal sequence analysis (Fig. 5).

The present procedures minimize autolysis of these enzymes during isolation and avoid many of the difficulties previously encountered in the purification of neutral proteases (14,15). Furthermore, two previously unrecognized species of the neutral protease from B. subtilis are separated. Although the molecular basis of the difference in specific activity and chromatographic behavior of neutral proteases A and B has not yet been clarified, the fraction which binds more tightly to the affinity column catalyzes the hydrolysis of FAGLA at a

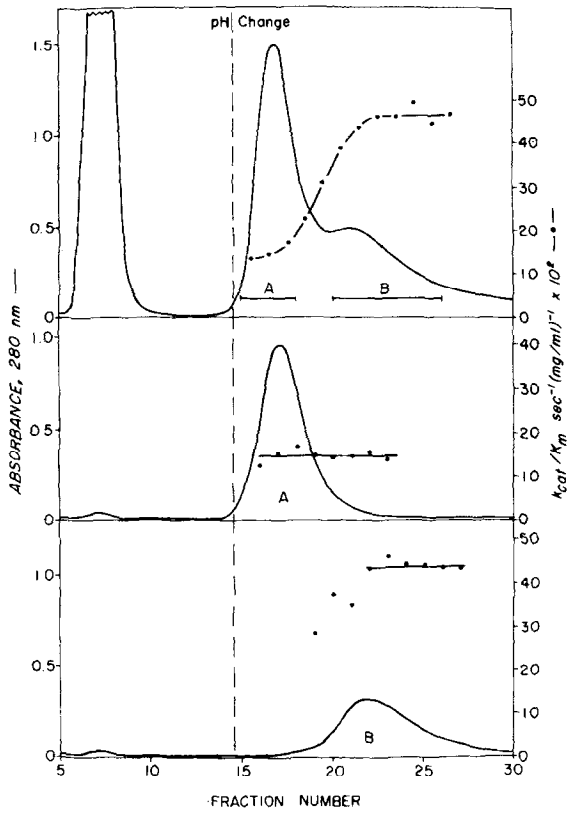


Figure 4: Affinity chromatography of crude neutral protease (1.4 g) on a column of Adsorbent 2 (2.5 x 17.5 cm). The column was equilibrated as in Fig. 2 and eluted at pH 6.3 with 100 mM NaCl, 10 mM $CaCl_2$ containing 50 mM MES. Fractions of 15.5 ml were collected at 100 ml/hr and pooled as indicated in the upper diagram. The results of rechromatography of the pooled fractions (A and B) are shown in the lower diagrams. The specific activity pertains to FAGLA hydrolysis.

higher rate. Differences in the substrate binding sites of the two enzymes could account for these observations.

Since the neutral metalloendopeptidases from two bacterial sources could be purified with the same system of affinity chromatography, it may be expected that these procedures will be effective in the purification of other neutral metalloendopeptidases from a variety of biological sources.

ACKNOWLEDGMENTS

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TABLE I. Extinction Coefficients and Kinetic Constants of Thermolysin and of Neutral Proteases A and B. The enzymes were purified by affinity chromatography (Fig. 1A and Fig. 4). The kinetic constants pertain to the hydrolysis of FAGLA.

| Enzyme | $E^{1\%}_{280}cm^{-1}$ | $\frac{k_{cat}/K_m}{sec^{-1}(mg/ml)^{-1}}$ |
|---|---|---|
| Thermolysin | 17.6 | 0.60 |
| Neutral protease A | 14.8 | 0.15 |
| Neutral protease B | 14.7 | 0.44 |

Neutral Proteases

A: ALA-ALA-THR-THR-GLY-THR-GLY-THR-THR-LEU-LYS-GLY-LYS-THR-VAL-

B: ALA-ALA-THR-THR-GLY-THR-GLY-THR-THR-LEU-LYS-GLY-LYS-THR-VAL-

Thermolysin: ILE-THR-GLY-THR-SER-THR-VAL-GLY-VAL-GLY-ARG-GLY-VAL-

Figure 5: Amino-terminal sequences of thermolysin and of the two neutral proteases isolated by affinity chromatography (Figs. 1A and 4). In each case a single sequence was observed by the technique of Edman and Begg (17) as adapted by Hermodson et al. (18). The sequence of thermolysin is identical with that reported by Titani et al. (19). Since sequence data from other portions of neutral protease indicate a homologous relationship with thermolysin (2), the sequences are aligned to give minimum base changes.

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