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AFFINITY CHROMATOGRAPHIC INTERACTIONS OF PROTEASES WITH LOW-MOLECULAR-WEIGHT SOYBEAN PROTEASE INHIBITORS

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SUMMARY

One of the five low-molecular-weight soybean protease inhibitors, protease inhibitor V (PI-V, Bowman–Birk inhibitor) with a molecular weight of 8000 daltons, is used for the affinity chromatographic purification of trypsin, chymotrypsin and kallikrein. The PI-V covalently bound to Sepharose 4B adsorbs bovine trypsin, chymotrypsin or porcine kallikrein at neutral pH. The adsorbed enzyme is eluted from the column with buffer containing a higher salt concentration and a lower pH. This low-molecular-weight PI-V affinity gel can separate trypsin and chymotrypsin from mixtures of these crude enzymes and can purify both enzymes to a homogeneous degree.

INTRODUCTION

Kunitz inhibitor, a soybean protease inhibitor with a molecular weight of 21,400 daltons, has been widely used to inhibit trypsin activity. Recently, this inhibitor, covalently linked to Sepharose 4B gel, has been employed to purify trypsin and chymotrypsin. It was used for affinity chromatography of trypsin by Mosolov and Lushnikova[1], Bartling and Barker[2], Porath and Sundberg[3], Liepnieks and Light[4,5], Sundberg and Porath[6], of chymotrypsin by Porath[7], of kallikrein by Fritz *et al.*[8] and of both trypsin and chymotrypsin by Amneus *et al.*[9]. This product is available also from commercial sources for purification of both trypsin and chymotrypsin[10].

We have described the isolation and purification of five low-molecular-weight (7000–8000 daltons) soybean protease inhibitors[11] and determined their usefulness as ligands for affinity chromatography of proteases[12]. Immunologic tests show that inhibitors I through IV are fully cross-reactive with each other but are

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distinct from inhibitor V (PI-V, Bowman–Birk inhibitor)*. Enzyme inhibition tests show that only inhibitor V inhibits both trypsin and chymotrypsin and is the most potent inhibitor for trypsin among them. For these reasons, PI-V may be useful as a ligand for the affinity purification of specific proteases. Since PI-V is only about a third the size of Kunitz inhibitor and can completely inhibit both crystalline bovine trypsin and chymotrypsin at an equimolar concentration, we believe that its use as a ligand would further increase the capacity of an affinity column for proteases. Furthermore, pepsin digestion of PI-V cleaves it into a tryptic and a chymotryptic inhibitor fragment, both of which can be separated by gel filtration chromatography[13]. Thus a specific affinity column for a single protease may be prepared. In the study reported here, we have observed the interaction of PI-V with trypsin, chymotrypsin and kallikrein by affinity chromatography and applied the procedure to the purification of these proteases from crude enzyme mixtures.

MATERIALS AND METHODS

Low-molecular-weight protease inhibitor PI-V was purified from the Tracy cultivar of soybean as previously described[11]. Crystalline bovine pancreatic trypsin (165 units/mg) and chymotrypsin (37 units/mg) were obtained from Worthington (Freehold, NJ, U.S.A.). TAME, BTEE, BAN, crude trypsin (10.4 units/mg in tryptic activity and 5.8 units/mg in chymotryptic activity) and porcine pancreatic kallikrein (2.5 units/mg based on BTEE activity) were purchased from Sigma (St. Louis, MO, U.S.A.). Sepharose 4B and Sephadex G-100 were Pharmacia (Uppsala, Sweden) products. All other chemicals and reagents used were of reagent grade.

Sepharose 4B gel was activated with cyanogen bromide, according to the method of Porath *et al.*[14]. Two and a half milligrams each of trypsin, chymotrypsin or PI-V were covalently conjugated to each millilitre of activated Sepharose 4B gel. About 100 ml of each type of gel were packed to a column (24 × 2.3 cm) for the chromatographic study. Each of the packed columns was washed with ten volumes of 1% ethanolamine, 0.1 *M* sodium borate buffer containing 1 *M* NaCl at pH 8.0 (buffer 2), 0.1 *M* sodium acetate buffer containing 1 *M* NaCl at pH 4.0 (buffer 3), buffer 3 adjusted to pH 2.0 with concentrated HCl (buffer 4) and finally each column was reequilibrated with pH 7.0, 0.05 *M* Tris–HCl buffer containing 1 m*M* $CaCl_2$ (buffer 1). All column elutions were carried out at 4°C in a cold room. Column flow-rates were adjusted to about 2 ml/min. Crystalline trypsin (50 mg), chymotrypsin (50 mg) or crude kallikrein (100 mg) was dissolved in 5 ml of buffer 1 for application to a PI-V–Sepharose 4B column (bed volume 100 ml). After the wash with three column volumes of buffer 1, the columns were eluted first with three volumes of buffer 2, then three volumes of buffer 3 and finally with three volumes of buffer 1 to re-equilibrate the column. For the separation of enzyme mixtures, 120 mg crude trypsin (containing 35% of chymotryptic activity) were applied to a PI-V–Sepharose 4B column (26 × 1.5 cm, bed volume 50 ml). Stepwise elution with buffers of different pH and salt concentrations was performed as described above.

* Abbreviations: TAME = *p*-tosyl arginyl methyl ester; BTEE = benzoyl tyrosyl ethyl ester; BAN = N-*tert.*-butyloxy-alanyl nitrophenyl ester; PI-V = Protease inhibitor V, Bowman–Birk inhibitor; SDS = sodium dodecyl sulfate.

The gradient elution procedure was accomplished with a linear gradient of three column volumes of pH 8.0 buffer (buffer 2) to three volumes of pH 4.0 buffer containing 0.5 *M* sodium acetate and 0.5 *M* calcium chloride. The affinity column could be regenerated with three volumes of buffer 4 and then equilibrated with ten volumes of buffer 1. The protein eluted from the column was monitored by measuring the absorbance of the eluate at 280 nm.

Tryptic activity was measured by the rate of hydrolysis of TAME as monitored by the increase in absorbance at 247 nm. Chymotryptic activity was measured by the rate of hydrolysis of BTEE as monitored by the changes in absorbance at 256 nm[15]. Elastase-like activity was measured by the rate of hydrolysis of BAN as monitored by the changes in absorbance at 347.5 nm[16]. Kallikrein activity was monitored by both BTEE and BAN assays and expressed as the change in absorbance per mg of enzyme in 10 min (*A* unit). Tryptic or chymotryptic activity was expressed as micromoles of TAME or BTEE hydrolyzed per minute.

Daily urine samples from six normal adults were pooled and concentrated from about 10 l to 40 ml with an Amican hollow fiber concentrator equipped with a cartridge for a molecular weight cutoff at 10,000 daltons. The concentrated sample was dialysed against buffer 1 and the enzymes purified by passage through the PI-V–Sepharose 4B column.

Fractions with kallikrein activity, eluted after the application of buffer 3, were pooled and concentrated in a Diaflo concentrator. Further purification was achieved by chromatography on a Sephadex G-100 column (157 × 0.9 cm). The fractions containing a single symmetric protein peak after gel filtration were pooled, dialysed and lyophilized for characterization by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate according to the method of Weber and Osborn[17].

RESULTS

The elution profile of 50 mg crystalline bovine trypsin on a PI-V–Sepharose 4B column is shown in Fig. 1a; the profile of 50 mg crystalline bovine chymotrypsin is depicted in Fig. 1b. In both Fig. 1a and 1b, the peak eluted by buffer 1 (peak I) was inactive. The peak eluted by buffer 2 (peak II) had less than 10% of the original specific activity. The protein peak eluted with pH 4.0 buffer (peak III) was very active. The fraction represented by the trailing end of peak III could not be completely eluted until the column was re-equilibrated with buffer 1 containing 1 m*M* of $CaCl_2$. More than 90% of the protein applied to the column was recovered in the eluate. Residual uneluted enzymes, if any, were removed from the column by washing with buffer 4 and then equilibrating with pH 7.0 buffer. The elution profile of 50 mg each of the PI-V on a trypsin–Sepharose 4B column (left) and on a chymotrypsin–Sepharose 4B column (right) is depicted in Fig. 2. PI-V was completely eluted by sequentially applying buffers 1, 2 and 3, including a final elution with buffer 4. Less than 20% of PI-V applied to the column was eluted with buffers 1 and 2. The main peak of PI-V was eluted with buffer 3. The elution profiles of PI-V on the trypsin column and chymotrypsin column were quite similar, suggesting that either enzyme can be used as a ligand for affinity purification of PI-V.

The elution profile of 100 mg of porcine pancreatic kallikrein (2.5 BTEE units/A_{280} protein unit) chromatographed on a PI-V Sepharose column is shown in Fig. 3.

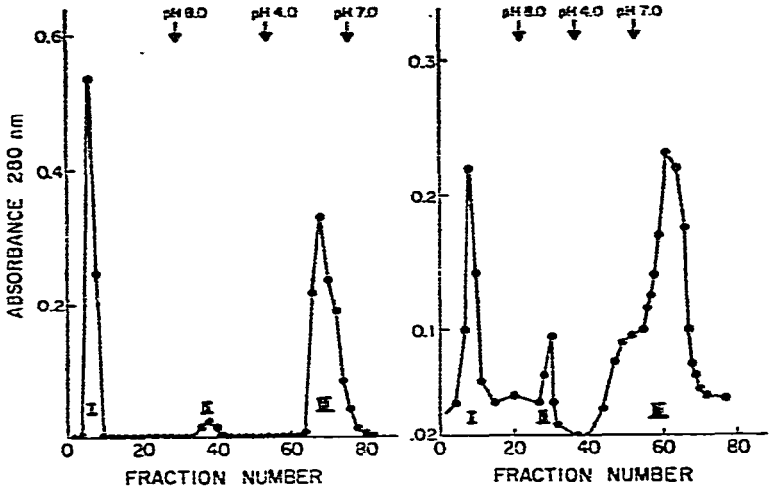

Fig. 1. Affinity chromatography of 50 mg of purified bovine trypsin on a PI-V–Sepharose 4B column (1a, left) and 50 mg of bovine chymotrypsin (1b, right) on the same column (100 ml). The arrow indicates the fraction at which buffer change occurred. Protein concentrations in the fractions were monitored by absorbance at 280 nm. Each fraction volume equals 10 ml.

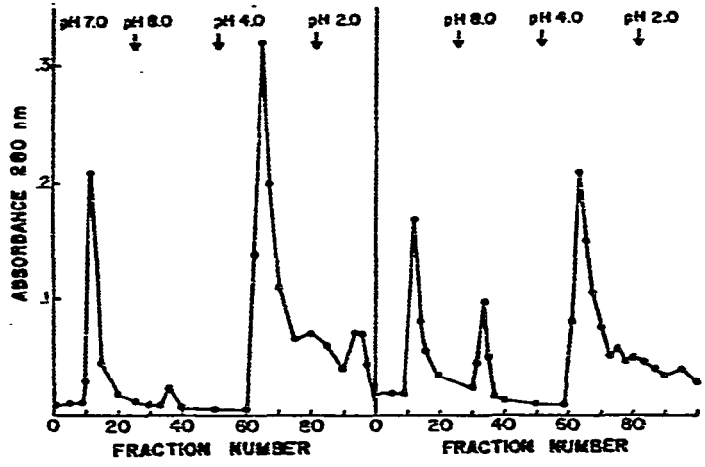

Fig. 2. Chromatography of 50 mg of purified PI-V each on a trypsin–Sepharose 4B column (left) and on a chymotrypsin–Sepharose 4B column (right) (both 100 ml). A pH 2.0 buffer was added to the elution profile before re-equilibration to ensure complete removal of PI-V from the column. Protein concentrations in the fractions were monitored by absorbance at 280 nm. Each fraction contains 10 ml.

The unadsorbed peak contained 50% of the protein applied but, as compared to the original sample, had less than 1% of the enzyme activity toward BTEE and BAN. The enzyme eluted with buffer 2 represented approximately 1% of the protein applied to the column. Compared to the original unfractionated sample, the main peak of activity eluted after the application of buffer 3 possessed a ten to twenty-fold

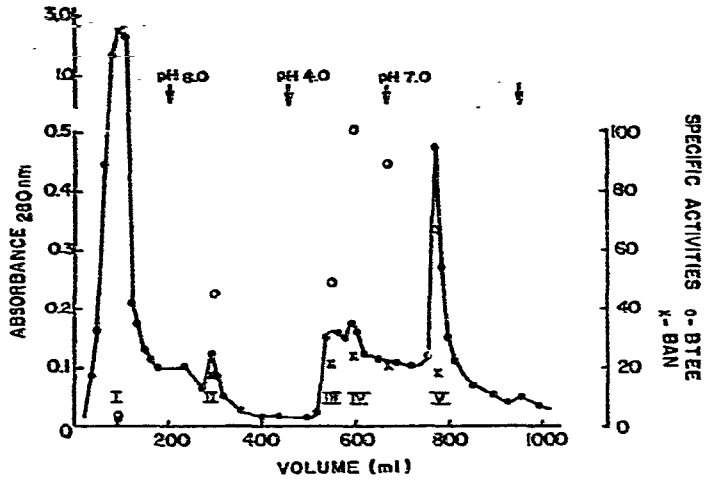

Fig. 3. Chromatography of 100 mg of purified porcine pancreatic kallikrein on a PI-V–Sepharose 4B column (100 ml). Specific activity toward BTEE (open circle) is expressed as change in absorbance at 256 nm in 10 min per A_{280} units of protein added. Specific activity toward BAN (×) is expressed as change in absorbance at 347.5 nm in 10 min per A_{280} units of protein added.

higher specific activity based on the BTEE assay. In contrast, the enzyme activity toward BAN increased only two-fold in peaks III, IV and V.

When crude human urine concentrate containing kallikrein was chromatographed on a PI-V–Sepharose 4B column (Fig. 4), the unadsorbed dark brownish fractions (peak I) contained no detectable enzyme activity toward either BTEE, BAN or TAME. The fractions eluted with buffer 2 showed low activity toward BTEE

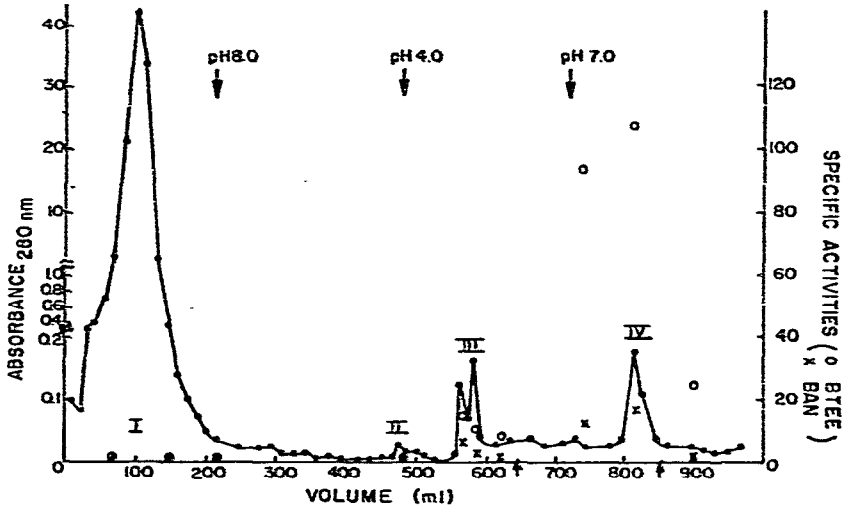

Fig. 4. Chromatography of concentrated daily urine from six normal adults on a PI-V–Sepharose 4B column (100 ml). Ten-ml fractions were collected. Fractions eluted from 630 ml to 850 ml were pooled, concentrated and chromatographed on a Sephadex G-100 column. Specific activity is expressed as in the legend of Fig. 3.

(1.2 units); the activity toward BAN was too low for measurement. The fractions eluted after the application of buffer 3 (peak III) exhibited appreciable kallikrein activity even though the protein concentration in these fractions, as measured by absorbance at 280 nm, was less than 0.2 *A*. When the column was re-equilibrated with buffer 1 (peak IV), an active peak toward BTEE (106 units) and BAN (17 units) was recovered. The activity ratio toward these two substrates was constant and was similar to that of a porcine pancreatic kallikrein repurified by the same procedure (Fig. 3).

The elution profile of 120 mg crude pancreatic trypsin (containing 35% chymotrypsin) on a PI-V–Sepharose 4B column (bed volume 50 ml) is shown in Fig. 5. Using a stepwise elution profile, the chymotrypsin was eluted with buffer 2 while the trypsin was retained on the column until the application of buffer 3. The two enzymes, therefore, were well separated. Chymotrypsin and trypsin activities were increased twelve-fold and sixteen-fold respectively, as shown in Fig. 5, left. Both enzymes were eluted as multiple peaks through the gradient when a second run with a pH and salt gradient elution was conducted. The major peak of both enzymes emerged after the application of buffer 3 (pH 4.0) at the completion of gradient elution. A seven-fold purification of chymotrypsin and twelve-fold purification of trypsin were observed even though the two enzymes were not separated.

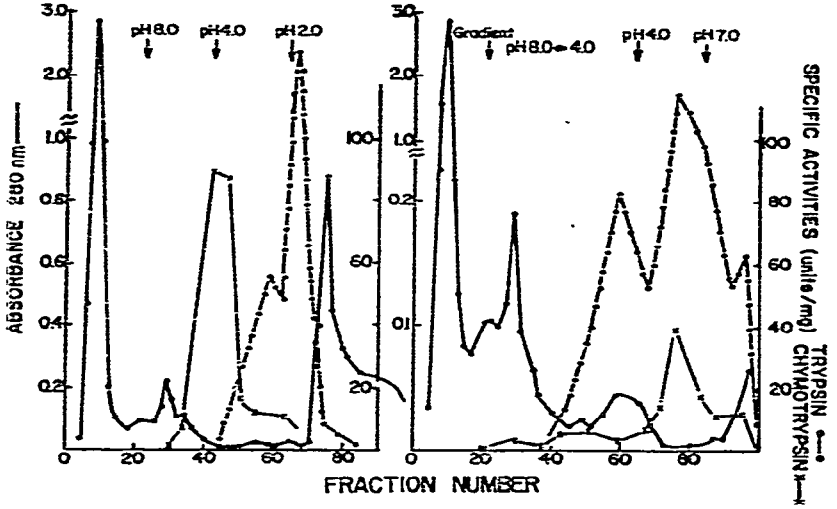

Fig. 5. Chromatography of 120 mg crude pancreatic trypsin (containing 35% chymotrypsin) on a PI-V–Sepharose 4B column (50 ml) by stepwise elution (left) and gradient elution (right). Fraction volume equals 7 ml. Specific activity of trypsin is expressed as micromoles TAME hydrolysed per min per mg protein added. Chymotrypsin activity is expressed as micromoles BTEE hydrolysed per min per mg protein added.

DISCUSSION

PI-V covalently linked to Sepharose 4B gel retains its affinity for both trypsin and chymotrypsin. The fact that the specific activity of neither crystalline trypsin nor purified chymotrypsin showed any further increase after affinity chromatography

implies that the crystalline enzymes were of high purity. However, substantial purification of a crude mixture of trypsin and chymotrypsin was seen. Since trypsin cleaves the PI-V molecule at the Lys-Ser bond (residues 16, 17) and chymotrypsin cleaves at the Leu-Ser bond (residues 43, 44) at equivalent molar ratio in solution, it is conceivable that the immobilized PI-V molecules on Sepharose 4B gel could have been modified by the proteases during the chromatographic procedures. Nevertheless, the binding capacity of the column remained unchanged after each run.

Purification of urinary protease can be achieved by one-step chromatography on a PI-V–Sepharose 4B column. The activity of this protease is similar to that of kallikrein based on substrate specificity. The most purified kallikrein exhibits only low activity toward TAME. The affinity of PI-V toward kallikrein is comparable to that of aprotinin, a low-molecular-weight pancreatic trypsin inhibitor which has been used for the affinity chromatography of urinary kallikrein[18].

In the stepwise elution procedure for the purification of crude enzymes, the chymotryptic and tryptic activities are well separated and both enzymes are recovered. The elution pattern indicated that the interaction of chymotrypsin with PI-V is affected by the binding of trypsin even though these two materials bind to separate sites on the PI-V molecule. A calcium and pH gradient elution profile shows the incomplete separation and partial loss of chymotryptic activity which may be due to denaturation of chymotrypsin at high calcium concentrations. Such an event would be responsible for the low recovery of chymotryptic activity. The recovery of tryptic activity, however, is increased as compared to that of the stepwise elution procedure.

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