Journal of Chromatography, 129 (1976) 97–105

CHROM. 9295

FRACTIONATION OF PROTEOLYTIC ENZYMES BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE AMINOCAPROYL PROFLAVIN

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(Received April 9th, 1976)

SUMMARY

The serine proteinases trypsin, chymotrypsin, elastase, and acrosin bind to the proflavin resin, the sulfhydryl proteinases ficin, bromelain, and papain are retarded by the resin, whereas most proteins and enzymes tested are not bound. Elution of the bound activities is accomplished by NaCl or by variation from the pH optimum of the enzyme. Commercially available enzymes that are bound or retarded are easily further purified by the column. The acrosin activity of sperm acrosomal extracts is separated into bound and unbound activities. Acrosin is purified 120-fold from sperm acrosomal extracts in a single step, yielding a specific activity of 96.

INTRODUCTION

The binding of proflavin (3,6-diaminoacridine) to the serine proteinases trypsin, thrombin, and chymotrypsin is accompanied by a substantial shift in the visible absorption spectrum. This spectral shift has been utilized by many investigators as a means of detecting complex formation and dissociation between proteinase, proflavin, and substrates or inhibitors[1–4]. Proflavin competitively inhibits the activity of several proteolytic enzymes, including trypsin[5,6], thrombin[4,7], and chymotrypsin[1,8–10]. The sulfhydryl proteinases papain and ficin show an enhanced activity toward substrates in the presence of proflavin[11–13]. The enhancement of enzyme activity appears to be due to proflavin binding at a site on the enzyme remote from the active site which contains the sulfhydryl group essential for catalytic activity[11,13–15].

We are interested in the use of affinity chromatography to purify enzymes localized in the acrosome of mammalian sperm. Some of these hydrolytic acrosomal enzymes have been shown to be necessary for sperm penetration of the egg during fertilization (for review, see McRorie and Williams[16]). We report here on affinity

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chromatography utilizing Sepharose aminocaproyl proflavin resin as a method for the separation of proteolytic enzymes. The procedure is applicable for purifying serine proteinases from natural materials or for removing serine proteinase contaminants from enzyme preparations. A preliminary report on the work has been presented[17].

EXPERIMENTAL

Materials

The following chemicals and biochemicals were obtained from the indicated companies. Proflavin (3,6-diaminoacridine) from Aldrich (Milwaukee, Wisc., U.S.A.); Bz-Arg-OEt (N-benzoyl-L-arginine ethyl ester), Bz-Tyr-OEt (N-benzoyl-L-tyrosine ethyl ester), and L-leucine *p*-nitroanilide from Sigma (St. Louis, Mo., U.S.A.); hippuryl-L-phenylalanine from K & K Labs. (ICN) (Plainview, N.J., U.S.A.); azocollagen (50–100 mesh) from Calbiochem (La Jolla, Calif., U.S.A.); bovine pancreatic trypsin, chymotrypsin, carboxypeptidase A, porcine pancreatic elastase, and wheat germ acid phosphatase from Worthington (Freehold, N.J., U.S.A.); Pronase (B grade) and porcine intestine peptidase (C grade) from Calbiochem; aminopeptidase M from Rohm & Haas (Henley, New York, N.Y., U.S.A.); papaya extract (crude papain powder type II from papaya latex), papain (twice crystallized from papaya latex), ficin (twice crystallized from fig tree latex), and bromelain extract (practical grade II from pineapple) from Sigma; bovine serum albumin (Fraction V) from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.); transferrin was a gift from D. Johnson of our department.

Preparation of resin

Sepharose aminocaproyl proflavin was prepared by a modification of the method of March *et al.*[18]. One hundred milliliters of Sepharose 4B that had been washed with glass distilled water were combined with an equal volume of 2 *M* Na_2CO_3. Cyanogen bromide was dissolved in dimethylformamide (100 g CNBr + 50 ml DMF) to give a concentration of about 1 g CNBr/ml. With vigorous stirring at 4°, 30 ml of the CNBr solution was added to the Sepharose and stirring continued for 1–2 min. The resin was collected on a plastic büchner funnel and washed with 10–15 volumes of cold 0.2 *M* $NaHCO_3$, pH 9.5. Two grams (1.5 mmoles) of 6-aminocaproic acid dissolved in 100 ml of the bicarbonate buffer were added to the washed resin and stirred at 4° overnight. The resin was collected on a funnel and washed with water. One gram (0.5 mmoles) of proflavin was dissolved with minimum HCl in 50 ml of water. 1.5 g 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.75 mmoles) dissolved in 100 ml of water was combined with the resin and proflavin solution and stirred at room temperature for 4 h. The resin was collected by filtration on a büchner funnel and washed with 0.1 *M* HCl and finally with water.

Detergent extraction of acrosin from rabbit sperm

To avoid the adsorption of acrosin on glass surfaces, plastic labware and siliconized quartz cuvettes were used throughout. All steps were at 3° unless indicated otherwise. The sperm acrosomal contents were extracted with detergent by a modification of the extraction procedure of Srivastava *et al.*[19]. One hundred epididymides

obtained on ice from Pel Freeze Biologicals (Rogers, Ark., U.S.A.) were flushed with cold Krebs' Ringer phosphate, pH 7.2, to give a volume of 60 ml of flushings. Sperm were sedimented by centrifugation at 1000 *g* for 10 min at 2° and then washed two times with 80 ml of Krebs' Ringer phosphate, pH 7.2. The washed sperm were suspended in 20 ml of 0.05 *M* Tris [tris(hydroxymethyl)aminomethane]–maleate, pH 6.1, 0.1% Hyamine 2389, 0.1% Triton X-100. The suspension was gently shaken for 90 min at 37°, and centrifuged at 12,000 *g* for 10 min. The resultant supernatant was dialyzed against 1 m*M* $CaCl_2$ overnight, and then concentrated against glycerol to yield a glycerol concentration of at least 50%. The glycerol concentrated acrosomal extract was stored (did not freeze) at –30°.

Protein determination

Protein concentration was estimated by the absorption at 280 nm assuming that 1 mg of protein per ml has an absorption at 280 nm of 1.0 in a 1-cm light path[20].

Enzymatic assays

All spectrophotometric assays were done at 22–23° with a Bausch and Lomb Spectronic Type 200 spectrophotometer utilizing a 1-ml siliconized quartz cuvette with a 1-cm light path. The rate of hydrolysis of Bz-Arg-OEt was measured by the method of Schwert and Takenaka[21] at 253 nm using a molar absorbance difference for Bz-Arg and Bz-Arg-OEt of 1150 M^{-1} cm^{-1} (determined with trypsin). Assays were performed in 0.05 *M* Tris–HCl, pH 8.6, containing 0.05 *M* $CaCl_2$ and 5×10^{-4} *M* Bz-Arg-OEt. Acrosin activity is expressed in International Units (μmoles per minute) using a molar absorbance difference of 1150 M^{-1} cm^{-1} for Bz-Arg-OEt hydrolysis and specific activity is expressed in units per mg of protein. Chymotrypsin activity was assayed according to the method of Hummel[22] by measuring the rate of hydrolysis of Bz-Tyr-OEt at 256 nm in 0.04 *M* Tris–HCl, pH 7.8, 0.05 *M* $CaCl_2$, 25% methanol (w/w) and 5.3×10^{-4} *M* Bz-Tyr-OEt. Carboxypeptidase A activity was followed by observing the hydrolysis of hippuryl-L-phenylalanine at 254 nm by the method of Folk and Schirmer[23] in 0.025 *M* Tris–HCl, pH 7.5, 0.5 *M* NaCl, and 1×10^{-3} *M* hippuryl-L-phenylalanine. Aminopeptidase M activity was measured at 405 nm by the method of Pfleiderer[24] in 0.06 *M* potassium phosphate, pH 7, containing 1.66×10^{-3} *M* L-leucine-*p*-nitroanilide. Proteolytic activity was measured by the hydrolysis of azocollagen. Twelve milligrams of azocollagen, enzyme, and buffer (0.2 *M* N-ethylmorpholine acetate, pH 8.6, 5 m*M* $CaCl_2$) were incubated in a final volume of 1 ml at 37° in a shaking water-bath for 30–120 min. After digestion 2 ml of water were added and the reaction mixture was filtered through Whatman No. 1 filter paper. The amount of azocollagen hydrolysis was measured by the enzyme-dependent increase in the absorbance at 520 nm of the filtrate. Elastase activity was assayed by observing the hydrolysis of *p*-nitrophenyl *tert.*-butyloxy-carbonyl-L-alaninate at 347.5 nm according to the procedure of Visser and Blout[25].

Acid activation

In order to dissociate rabbit acrosin from inhibitory component(s) that are present in acrosomal extracts[26] enzyme was incubated with an equal volume of 0.2 *M* acetic acid, 1 mg bovine serum albumin/ml at 37° for 10 min, then assayed for enzymatic activity as described above.

Chromatographic procedure

The protein (1–2 mg unless otherwise indicated) was applied to a 4 × 0.8 cm I.D. column of Sepharose aminocaproyl proflavin resin. The column buffer was 20 m*M* Tris–HCl, pH 8.6, 1 m*M* $CaCl_2$ for the enzymes and proteins in Table I, except that the following enzymes were applied to the column near their optimum pH: subtilisin (pH 6.0 and 8.0), bromelain and ficin (0.2 *M* sodium acetate, pH 5.0, 30 m*M* cysteine), papaya extract and papain [0.2 *M* sodium acetate, pH 6.0, 1 m*M* EDTA (ethylenediamine tetraacetate), 5 m*M* cysteine]. Acid phosphatase (20 m*M* sodium acetate, pH 5.3), peptidase (pH 8), and bovine serum albumin were assayed by their absorbance at 280 nm; transferrin was assayed by its absorbance at 420 and 280 nm.

RESULTS

Trypsin and chymotrypsin are known to be inhibited by proflavin and these enzymes are bound by the Sepharose aminocaproyl proflavin column (proflavin column). Fig. 1 shows the pH elution profile of commercially purified bovine trypsin on the proflavin column. Forty-eight per cent of the protein but none of the activity washes through the column. Stepwise elution with acidic pH elutes the enzyme in the pH range of pH 6.2–5.3. Bovine trypsin and chymotrypsin activities are readily separated by elution of the proflavin column with a NaCl gradient, as shown in Fig. 2. Typically about 45% of the protein (but no enzyme activity) washes through the proflavin column. A purification of about 1.8-fold is observed when commercially purified trypsin or chymotrypsin is chromatographed on the proflavin column.

Some enzymes neither wash through nor are bound by the proflavin column, but instead are retarded. Much of the hydrolytic activity toward Bz-Arg-OEt, and

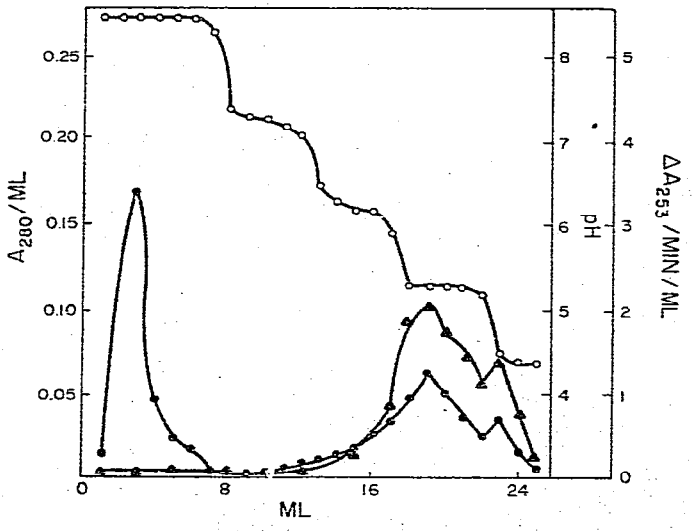

Fig. 1. Elution of trypsin from Sepharose aminocaproyl proflavin column by acidic pH. Buffers used were 50 m*M* Tris–HCl, 1 m*M* $CaCl_2$ (above pH 6) and 50 m*M* acetic acid-NaOH, 1 m*M* $CaCl_2$ (below pH 6). The hydrolytic activity toward Bz-Arg-OEt was assayed as described in Methods. ●, A_{280}; ○, pH; ▲, ΔA_{253}.

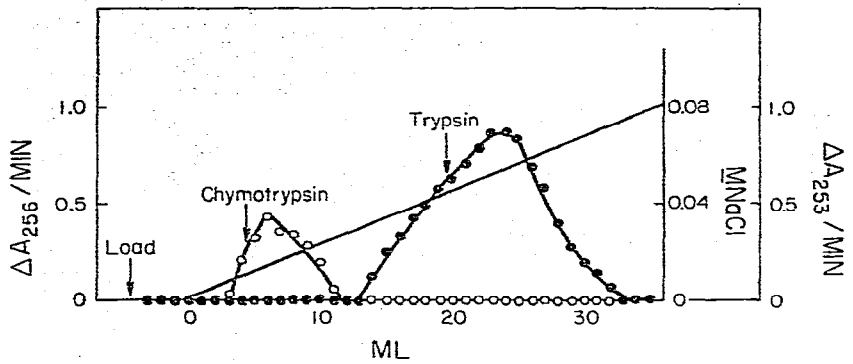

Fig. 2. Separation of trypsin and chymotrypsin activities by elution of the Sepharose aminocaproyl proflavin column with a NaCl gradient. A solution containing trypsin and chymotrypsin (0.5 mg of each) was loaded on the column. ●, ΔA_{253}; ○, ΔA_{256}; ———, M NaCl.

nearly all of the protein, in crude bromelain extracts washes through the proflavin column. Some Bz-Arg-OEt activity is retarded. Fig. 3A shows that some of the Bz-Arg-OEt activity, and very little of the protein, in papaya extracts, is also retarded by the proflavin column. The wash through activity in the column void volume is probably due to the presence of proteinases other than papain in papaya extracts. Papain (twice crystallized from papaya extracts) is retarded and purified about 1.3-fold by the proflavin column (Fig. 3B). Similarly, all of the Bz-Arg-OEt activity and some of the protein in ficin is retarded by the proflavin column (data not shown).

Table I shows the binding of several enzymes and non-enzymatic proteins to the proflavin column. Elastase, an endopeptidase containing a serine–histidine catalytic site, as do trypsin and chymotrypsin, binds to the proflavin column. The exopeptidases carboxypeptidase A and aminopeptidase M, and peptidase, are not bound. Pronase is a mixture of neutral and alkaline proteinases, aminopeptidases, and carboxypeptidases[27], and its proteolytic activity as measured by the rate of hydrolysis of azocollagen is partially bound. Subtilisin contains a serine–histidine active site, but is not inhibited by proflavin[8] and does not bind to the proflavin column. Recovery of the bound or unbound enzymes and proteins of 90% is achieved.

Acrosin is competitively inhibited by proflavin (data not shown). The pro-

TABLE I

BINDING OF SEVERAL PROTEINS TO SEPHAROSE AMINOCAPROYL PROFLAVIN

The enzymes were applied to the column in buffer at the enzymatic pH optimum, as described in Methods. Bound enzymes were eluted with buffer made 0.1 M in NaCl.

Not bound	*Retarded*	*Bound*
Acid phosphatase	Ficin	Acrosin
Aminopeptidase M	Papain	Chymotrypsin
Bovine serum albumin		Elastase
Carboxypeptidase A		60% of pronase activity
Peptidase		Trypsin
40% of pronase activity		
Subtilisin		
Transferrin		

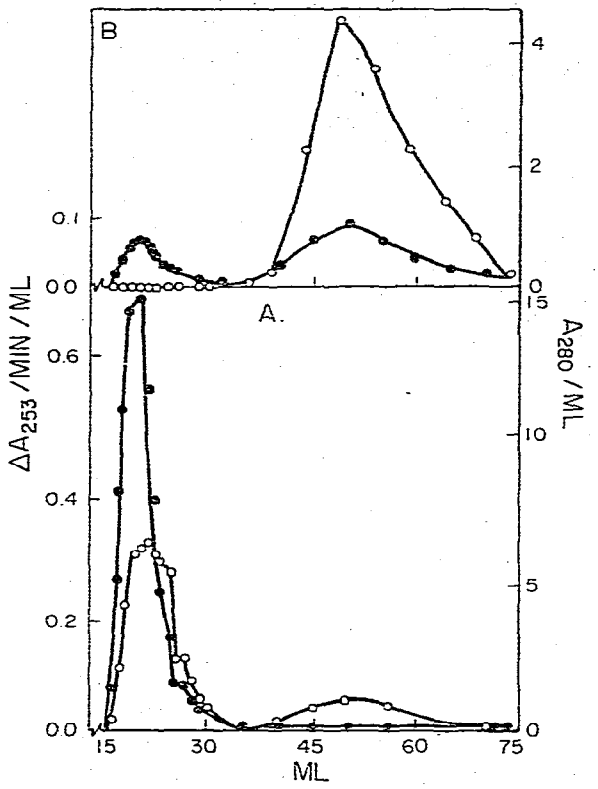

Fig. 3. Elution profile of papaya extract (A) and papain (B) using the Sepharose aminocaproyl proflavin column. The 10 × 1.4 cm I.D. column (column volume 15 ml) was loaded with papaya extract (total soluble absorbance at 280 nm = 20) or papain (total soluble absorbance at 280 nm = 80) and washed with 20 m*M* Tris–HCl, pH 8.6, 1 m*M* $CaCl_2$. ●, A_{280}; ○, ΔA_{253}.

teolytic activity in sperm acrosomal extracts is fractionated by the proflavin column, as shown in Fig. 4. Eighty-three per cent of the protein and some of the acrosin activity washes through the column. The major portion of acrosin binds to the column, and is fractionated into two species by stepwise elution with NaCl. The unbound acrosin activity, and the acrosin eluted with 0.05 *M* NaCl (inhibited acrosin) have very low Bz-Arg-OEt activity, but pre-incubation at acidic pH increases activity about tenfold. The 0.3 *M* NaCl-eluted acrosin activity (active acrosin) is unaffected by pre-incubation at acidic pH and has a specific activity of 96. This specific activity represents a 120-fold purification from the crude acrosomal extract by a single column chromatography step. Both the inhibited and active forms of acrosin have good proteolytic activity, as shown by their hydrolysis of azocollagen. The recovery of acrosin activity by the proflavin column procedure is about 95%.

DISCUSSION

The data in Table I indicate that serine proteinases that are competitively inhibited by proflavin are most tightly bound to the proflavin column, those enzymes

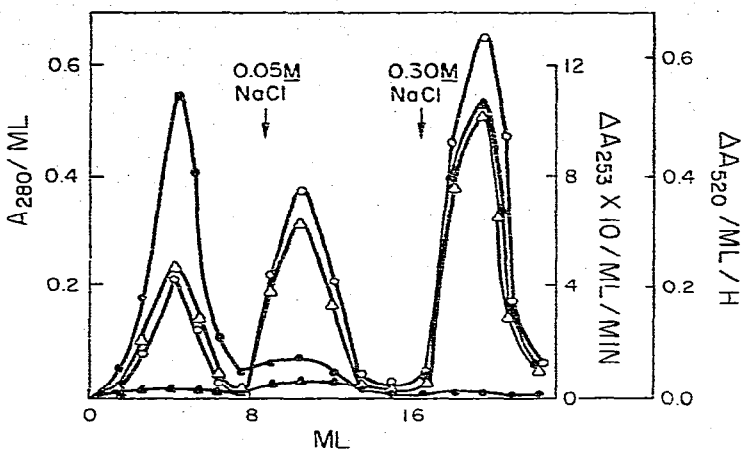

Fig. 4. Fractionation of rabbit acrosomal extract by chromatography on Sepharose aminocaproyl proflavin. Fifteen milligrams of acrosomal extract were applied to the 8 × 1.0 cm I.D. column. The starting buffer (20 m*M* Tris–HCl, pH 8.6, 1 m*M* $CaCl_2$) was made 0.05 *M* in NaCl and 0.30 *M* in NaCl as indicated to elute the bound activities. ●, A_{280}; ○, ΔA_{520}; ▲, ΔA_{253}; △, ΔA_{253} after acid activation.

that are activated by proflavin (the sulfhydryl proteinases ficin and papain) are slightly retarded by the column, and finally those enzymes that are unaffected by proflavin (subtilisin, etc.) are not bound. Elution of the bound enzymes can be accomplished by variation from pH optimum of the enzyme or by increasing the salt concentration, or a combination of both, depending on the stability or properties of the enzyme under investigation.

Uses of the proflavin column include purification of bound or retarded proteinases from natural materials or the removal of such proteinase contaminants from enzyme preparations. Bound proteinases can be fractionated, as shown for trypsin and chymotrypsin in Fig. 2.

In the course of these studies other ligands and spacer groups were investigated. In a few experiments acriflavin was substituted for the proflavin ligand, and similar binding of trypsin was observed. Wilchek (personal communication) has suggested that hydrazides may be superior spacer groups due to the reduced charge of the hydrazide–agarose linkage as compared to the charge created when CNBr coupling of agarose and the spacer group is utilized. In the present study the use of adipic monohydrazide and adipic dihydrazide in place of 6-aminocaproic acid as a spacer group gave similar binding of trypsin.

Our laboratory procedure is to pour a column of newly prepared resin, pass crude extract over it, and wash with eluting solution. The column is now ready for use. The column is very stable, and has been used repeatedly for nine months. Although the column is stored in the refrigerator between runs, this is not necessary since proflavin is a bacteriostatic agent.

There have been several reports dealing with the purification of various proteolytic enzymes by affinity chromatography. The ligands used include antibodies, naturally occurring inhibitors, substrates or their analogs, competitive inhibitors, and molecules that bind enzyme by virtue of their hydrophobicity.

The unique features of the proflavin resin are that all serine proteinases (except subtilisin, which is unlike other serine proteinases in several respects[28]) bind to the column, and that elution does not require pH change, organic solvents, or substrate or inhibitor.

Acrosin is a serine–histidine endoproteinase that is similar to trypsin in many respects (although there are some important dissimilarities, see McRorie and Williams[16] for review), and is competitively inhibited by proflavin. Acrosin (the 0.3 *M* NaCl-eluted enzyme activity) is purified 120-fold from acrosomal extracts by the proflavin column in a single step. The resulting specific activity of 96 on Bz-Arg-OEt compares favorably with previously reported specific activities of 13 (calculated) for partially purified rabbit acrosin[29] and 20 for purified rabbit acrosin[30]. Acrosin has been purified from other species by the more laborious purification techniques utilizing column chromatography on ion-exchange and gel filtration resins to specific activities of 90 (calculated) from boar[20] and 44 from human[31] sperm. Fritz *et al.*[32] have utilized affinity chromatography on benzamidine cellulose to purify boar acrosin to a specific activity of 165, and Garner and Cullison[33] have partially purified bovine acrosin to a specific activity of 11 on agarose–Gly-Gly-Tyr(O-benzoyl)-Arg, as assayed using Bz-Arg-OEt.

The calculated specific activities listed above were calculated from references 29 and 20 using a molar absorbance difference of 1150 M^{-1} cm^{-1} for the hydrolysis of Bz-Arg-OEt. As shown by previous workers[26], acrosomal extracts from rabbit epididymal sperm contain both free acrosin and acrosin complexed with inhibitor. The Sepharose proflavin resin separates the inhibited and active forms of acrosin. Work is presently in progress to characterize the unbound and bound acrosin species, which appear to have different properties.

ACKNOWLEDGEMENTS

We acknowledge the excellent technical assistance of Ms. Silvia Halfon. This work was supported by NIH Contracts 69-2103 and 70-2147 awarded by the U.S. Public Health Service, by Ford Foundation Grant 680-0805A, and by NIH Postdoctoral Fellowship (5 F22 HD02580-02) to J.H.B.

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