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Note

Partial purification of bovine acrosin by affinity chromatography

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The spermatozoan acrosomes of several species contain a trypsin-like proteinase that has been implicated in the process of fertilization[1–5]. This acrosomal proteinase, named acrosin[2,6], has been fractionated into several molecular forms and may represent a mixture of isozymes[3,5,7]. Acrosomal extracts obtained from bovine spermatozoa contain at least three major fractions possessing trypsin-like activity[5,7]. Effective characterization of acrosin has been hampered by the difficulty of isolating adequate amounts of the various fractions.

The work described in this article was carried out primarily to develop a simple method for partial purification of bovine acrosin that would facilitate subsequent isolation of the individual molecular forms of the enzyme. A simple procedure for direct purification of acrosin is described.

MATERIALS AND METHODS

Preparation of acrosomal materials

Acrosomal materials were obtained from bovine spermatozoa by detergent treatment. For each purification series, several bovine ejaculates (approximately 50 ml of semen) were diluted with two volumes of Krebs–Ringer phosphate buffer (pH 7.0) (ref. 8) containing 0.5% fructose (KRPF). The spermatozoa were separated from the diluted seminal plasma by centrifugation (1250 × *g* for 45 min) through a stepwise gradient of 5, 10, and 15% Ficoll (Pharmacia, Piscataway, N.J., U.S.A.) in KRPF. The resultant spermatozoan pellet was washed with KRPF before incubation for 1 h at 37° in KRPF containing 0.15% Hyamine 2389 (Rohm and Haas, Philadelphia, Pa., U.S.A.) for acrosomal removal[9]. The dislodged acrosomal material remaining in the supernatant following each of two different centrifugations (1500 × *g* for 5 min) was pooled and frozen in liquid nitrogen (—196°). This crude material was designated acrosomal extract.

Chromatographic system

The purification system for bovine acrosin was an affinity column consisting of agarose–Gly–Gly–Tyr(O-benzyl)–Arg (Miles Labs., Kankakee, Ill., U.S.A.). The agarose gel was stabilized against compaction with 3-mm glass beads[10]. The chromatographic agent (10-ml gel volume) and glass beads were simultaneously added in small increments to a 9 mm × 300 mm column. The packed column was equilibrated with

10 m*M* phosphate buffer (pH 7.5) before a sample was applied. The acrosomal extracts were thawed immediately before application to the column. Following washing of the column with the equilibration buffer to remove the unadsorbed proteins, the proteinase was released with 5 m*M* HCl (pH 2.6). All chromatographic procedures were carried out at 5°. Polypropylene containers were used whenever possible to avoid loss of material by adherence to glassware. In addition, all glassware was siliconized (Siliclad; Becton, Dickinson and Co., Parsippany, N.J., U.S.A.) before use.

Electrophoretic fractionation

The crude acrosomal extracts and purified materials were fractionated in a miniature (2-mm-I.D. columns) electrophoretic system at pH 4.3 (ref. 11). Each column was stopped when the ionic front had migrated 35 mm into the separating gel. Observation of the ionic front was enhanced by placing a fluorescent light directly behind the electrophoretic apparatus during the fractionation procedure. Migration was monitored using the light diffraction produced by the ionic front.

Activity measurements

The trypsin-like esterolytic activity of acrosin on N-α-benzoyl-L-arginine ethyl ester (BAEE) was measured spectrophotometrically[12]. The concentration of protein was measured by the method of Lowry *et al.*[13]. Proteinase activity was detected in polyacrylamide gels following electrophoretic fractionation using the chromogenic substrate α-N-benzoyl-DL-arginine-β-naphthylamide (BANA) and the diazonium salt, Fast Garnet GBC[7]. This chromogenic staining system was also used for qualitative estimation of BANA-amidohydrolase activity in the various fractions obtained as chromatographic effluent. Naphthol blue black was used to stain for protein fractions in the polyacrylamide gels[14].

Molecular weight estimates

Estimates of the molecular weights of the protein fractions of partially purified bovine acrosin were obtained using a miniature (2-mm-I.D. columns) dodecyl sulphate electrophoretic system employing 10% acrylamide in the separating gel[15]. The protein solutions were incubated at 90° for 1 min in 1% dodecyl sulphate and reduced by the addition of β-mercaptoethanol before application onto the gels[15]. The standards used for calibration were ovalbumin, aldolase (Pharmacia), bovine serum albumin, and bovine pancreatic trypsin (Sigma, St. Louis, Mo., U.S.A.).

RESULTS AND DISCUSSION

Affinity chromatography

Acrosin was separated from the bulk of proteinaceous material in bovine acrosomal extracts using an affinity column consisting of agarose–Gly–Gly–Tyr(O-benzyl)–Arg. As shown in Fig. 1, acrosin was released from the washed column with 5 m*M* HCl. Examination of BAEE hydrolysis rates in the acrosomal extracts, the chromatographic effluent during washing, and the eluate released with 5 m*M* HCl indicated that utilization of this chromatographic system resulted in a 22-fold purification of acrosomal proteinase. As shown in Table I, the specific activity of the acrosin

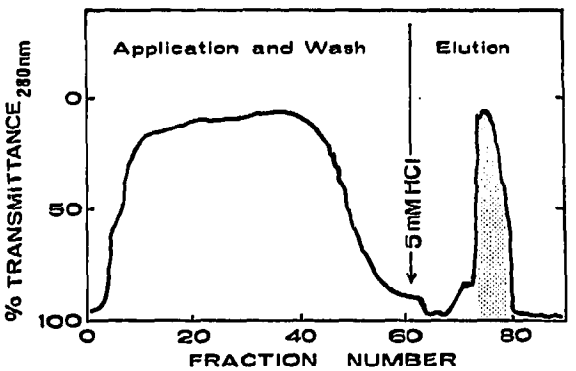

Fig. 1. Affinity chromatography of bovine acrosin on agarose–Gly–Gly–Tyr(O-benzoyl)–Arg. Twenty millilitres (100 mg protein) of acrosomal extract were applied to the column. The column was washed with 10 m*M* phosphate buffer (pH 7.5) before release of acrosin with 5 m*M* HCl (pH 2.6). Each fraction was 1.2 ml. Fractions 72–79 (shaded area) were found to contain acrosin activity and were pooled for subsequent analyses.

purified by this system was usually around 11 μmoles BAEE hydrolysed/min/mg protein.

Fig. 2 shows the electrophoretic patterns of acrosomal proteins obtained at various stages of purification. It can be seen that the crude extract contained at least eighteen proteins, whereas after affinity chromatography the number was reduced to about five fractions. Zymographic analyses of this material showed that all three major proteinase fractions present in acrosomal extracts were eluted from the affinity column after increasing the hydrogen ion coencentration of the buffer (pH 2.6). Zymographic evaluation of each of the fractions within the enzyme peak suggested no difference in affinity of the individual proteinase forms.

This study indicates that bovine acrosin can be partially purified directly from acrosomal extracts by affinity chromatography. The chromatographic agent, agarose–Gly–Gly–Tyr(O-benzoyl)–Arg, has been previously utilized for purification of papain[16,17]. Adaptation of this chromatographic system for partial purification of acrosin

TABLE I

PURIFICATION OF BOVINE ACROSIN

Trypsin-like esterolytic activity detected in materials obtained during purification of acrosomal proteinase. Enzyme activity is expressed as the mean BAEE hydrolysis rate ± S.D. for seven different purification trials. Detergent extracts of ejaculated spermatozoa were applied to a 9 mm × 300 mm column containing agarose–Gly–Gly–Tyr(O-benzoyl)–Arg. The column was washed with 10 m*M* phosphate buffer (pH 7.5) and all fractions containing unabsorbed proteins were pooled and designated "wash". The proteinaceous material released from the washed column with a change to 5 m*M* HCl (pH 2.6) was designated chromatography eluate.

No.	*Fraction*	*Enzyme activity*		*Purification (fold)*
		Units/ml	*Specific activity*	
1	Detergent extract	2.5 ± 0.7	0.5 ± 0.1	1
2	Wash	1.0 ± 0.5	0.5 ± 0.2	–
3	Chromatography eluate	11.2 ± 2.8	10.9 ± 2.1	22

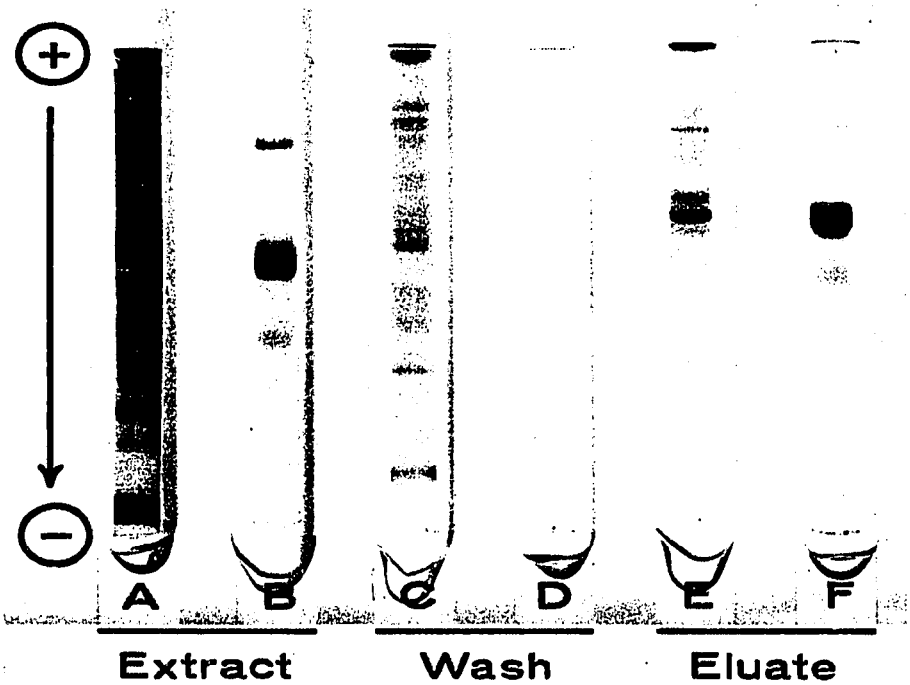

Fig. 2. Electrophoretic patterns of bovine acrosomal materials obtained at various stages during purification of acrosin. Migration in the electrophoretic system (pH 4.3) was from top (anode) to bottom (cathode). Gels A, C, and E were stained for protein with naphthol blue black, whereas the corresponding identical gels B, D, and F were stained for proteinase activity as described in the text.

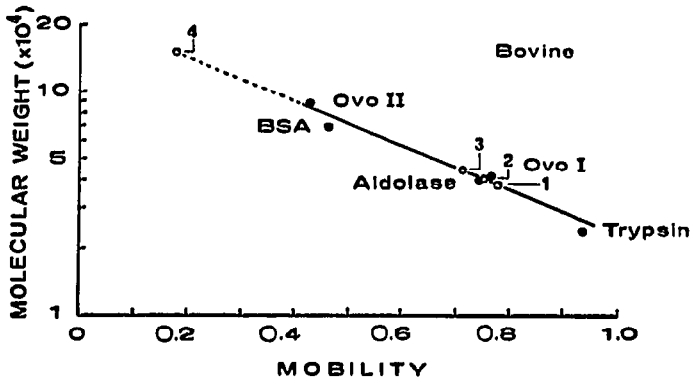

Fig. 3. Estimation of the molecular weights of the protein fractions (1, 2, and 3) of partially purified bovine acrosin using dodecyl sulphate electrophoresis. The three principal fractions had molecular weights of 34,000, 37,000, and 44,000. A fourth component (4) had an approximate molecular weight of 155,000. The protein standards were trypsin (23,800), ovalbumin monomer (45,000), aldolase (40,000), ovalbumin dimer (90,000), and bovine serum albumin (67,000). The experimental details are given in the text.

should facilitate subsequent isolation of individual molecular forms by other purification methods. It should be noted that during preparation of this manuscript an individual form of boar acrosin has been highly purified using an affinity column of benzamidine-cellulose following partial purification of acid-extracted acrosomal material by gel filtration[18].

Molecular weight estimates of the fractions purified by affinity chromatography are shown in Fig. 3. The results of three determinations indicated that the average molecular weights of the three major protein fractions were 34,000, 37,000 and 44,000 daltons. An additional fraction of approximately 155,000 daltons was noted also. These initial estimates of molecular weight may not represent the active components purified by affinity chromatography because treatment with β-mercaptoethanol would result in dissociation if the active fractions were composed of subunits. This study, however, does provide the groundwork for more definitive characterization of each of the active forms of bovine acrosin.

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