Studies on Ram Acrosin

ACTIVATION OF PROACROSIN ACCOMPANYING THE ISOLATION OF ACROSIN FROM SPERMATOOZOA, AND PURIFICATION OF THE ENZYME BY AFFINITY CHROMATOGRAPHY

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1. A previously described, freeze-dried, partially purified ram acrosin preparation was fractionated on a column of Sepharose linked to the acrosin inhibitor *p*-(*p*'-aminophenoxy-propoxy)benzamidine. Two acrosin fractions were obtained. 2. β -Acrosin was homogeneous, quite stable at low pH and very stable when freeze-dried. Its molecular weight is about 38 000, and it contains about six sugar residues per molecule, but no sialic acid. ψ -Acrosin consisted of at least three unstable forms of acrosin. 3. When the entire purification process, starting from collection of semen, was carried out as rapidly as possible, the yield of β -acrosin was increased and very little ψ -acrosin was obtained. 4. In fresh ram semen the acrosin is present as the intra-acrosomal zymogen, proacrosin. After its extraction from spermatozoa autoproteolytic reactions convert proacrosin into β -acrosin; ψ -acrosin appears to be breakdown products of β -acrosin. 5. When β -acrosin was passed through a column of Sepharose linked to the non-inhibitory deamidinated analogue of the inhibitor it behaved as a hydrophobic protein. This is consistent with our view that acrosin (as zymogen) occurs in spermatozoa as a membrane-bound protein. 6. Success in the isolation of pure acrosin in high yield calls for an affinity adsorbent with the appropriate subsidiary hydrophobic properties.

We have adduced evidence that the trypsin-like serine proteinase acrosin (EC 3.4.21.10) of ram spermatozoa is bound to the inner acrosomal membrane (Brown & Hartree, 1974, 1976; Brown *et al.*, 1975a) and that it is this bound form of acrosin that plays a role in the penetration of a spermatozoon into a mammalian egg (Brown & Hartree, 1976; Hartree, 1977). Acrosin is released in soluble form from spermatozoa at pH values below 3 or by Ca^{2+} (Fritz *et al.*, 1972; Brown & Hartree, 1974, 1976). In previous papers we described a partial purification of solubilized ram acrosin (Brown *et al.*, 1975a) and a fluorimetric method for determining the molar concentration of active centres (operational molarity) of an acrosin solution (Brown *et al.*, 1975b).

An inactive precursor of acrosin, proacrosin, was first detected in the seminiferous tubules of rabbit testis (Meizel, 1972). It has since been isolated from spermatozoa of rabbit, bull, ram and boar (Meizel & Mukerji, 1975; Shams Borhan *et al.*, 1976; Polakoski & Parrish, 1977). In freshly collected semen from these species acrosin is mainly, and possibly entirely, present as the inactive precursor. However, activa-

tion can occur rapidly. In the present paper we describe the isolation from ram spermatozoa of a stable, pure, soluble form of acrosin, and the activation of proacrosin that occurs during purification. We also give information on the amino acid composition and the molecular weight of the product. As a protein, soluble ram acrosin is more hydrophobic than trypsin. This is consistent with our view that acrosin is a peripheral membrane component.

Materials

Affinity adsorbents

The benzenesulphonate of *p*-(*p*'-aminophenoxy-propoxy)benzamidine (I) was prepared by the method of Baker & Erickson (1968). The yield can be improved by modifications that were introduced by Jameson & Elmore (1974).

All melting points are uncorrected.

p-(Phenoxypropoxy)nitrobenzene. Phenol (1.88 g), 5.20 g of 3-bromopropoxy-*p*-nitrophenol (Gagnon *et al.*, 1952) and 2.88 g of powdered K_2CO_3 were stirred with 50 ml of formdimethylamide at 100°C for 11 h. The solid was filtered off and the filtrate evaporated *in vacuo* to a syrup. This was shaken with water and chloroform, a little ethanol being added to break the

Abbreviations used: SDS, sodium dodecyl sulphate; Bz-Arg-OEt, *N*- α -benzoyl-L-arginine ethyl ester.

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emulsion. The chloroform layer was washed twice with water, dried with Na_2SO_4 and evaporated. The product was crystallized twice from ethanol/water (1:1, v/v) and dried over CaCl_2 [yield 3.40g (62%); m.p. 67–70°C]. A sample recrystallized from 2-methylpropan-2-ol/propan-2-ol (5:1, v/v) had m.p. 69–70°C (Found: C, 65.9; H, 5.5; N, 5.3. $\text{C}_{15}\text{H}_{15}\text{O}_4\text{N}$ requires C, 65.9; H, 5.5; N, 5.1%).

p-(Phenoxypropoxy)aniline hydrochloride (II). The nitro compound (2.73g), in 200ml of ethanol, was mixed with 0.5g of palladized charcoal (5% Pd) and stirred for 5h in H_2 at 2atm (200kPa) pressure. (During this period the A_{350} value of a filtered sample decreased to 5% of the initial value.) The charcoal was filtered off, the filtrate evaporated to about 10ml, and 0.25M-HCl added until the colour changed from brown-purple to grey-green. The precipitated hydrochloride was redissolved by heating; on subsequent cooling to 0°C it crystallized as grey needles. These were collected, washed with cold 0.1M-HCl and dried *in vacuo* over H_2SO_4 and KOH [yield 2.24g (80%); m.p. 140–143°C after previous sintering]. Recrystallization from 0.1M-HCl (charcoal) yielded fine pale-grey needles, m.p. 143–145°C after sintering at 137°C (Found: C, 64.4; H, 6.4; N, 5.1; Cl, 12.6. $\text{C}_{15}\text{H}_{17}\text{O}_2\text{N}\cdot\text{HCl}$ requires C, 64.4; H, 6.5; N, 5.0; Cl, 12.7%).

- (I) $\text{R} = \text{C}(\text{NH})\text{NH}_2$
 (II) $\text{R} = \text{H}$

Sepharose-compound (I) and Sepharose-compound (II) (adsorbents I and II). Our standard method is derived from that used by Jameson & Elmore (1974) for the preparation of adsorbent (I). The essential change is a stricter control of the activation step. Sepharose 4B (50ml of suspension) was washed twice, by centrifugation at 100g, with 2M- Na_2CO_3 . It was resuspended in 50ml of water and diluted to 100ml with 2M- Na_2CO_3 . The suspension was cooled to 9°C, stirred, and treated with a solution of 2.5g of CNBr in 5ml of acetonitrile (Marsh *et al.*, 1974). Stirring was continued for exactly 2min, during which time the temperature rose to 11°C. The suspension was transferred rapidly to a 7cm-diameter sintered-glass Buchner funnel (porosity 3) to which a slight vacuum was applied. The gel was washed rapidly with 800ml of ice-cold 0.1M- NaHCO_3 . The washed gel was suspended in 50ml of 0.1M-sodium acetate buffer, pH 5, and adjusted to pH 6 with 1M-acetic acid. To this suspension was added, as a solution in 5ml of aq. 50% (v/v) methanol, either 50mmol of compound (I) (22.2mg) or 50mmol of compound (II) (14.0mg). The mixture was stirred very gently for 18h at 20–

22°C, then transferred to a chromatography tube (4cm²) and washed with sodium acetate and NH_4Cl buffers as described by Jameson & Elmore (1974). After a final wash with 200ml of water, the adsorbent was stored in 3mM- NaN_3 at 5°C.

Reagents and proteins for polyacrylamide disc-gel electrophoresis, SDS/polyacrylamide disc-gel electrophoresis, gel filtration (exclusion chromatography) and ion-exchange chromatography

Marker proteins, for measurements of molecular weight, were supplied by Becton Dickinson (U.K.) Ltd., Wembley, Middx., U.K., except for soya-bean trypsin inhibitor, which was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. *NN'*-Methylenebisacrylamide (Eastman) was from Kodak, Liverpool, U.K. Bovine serum albumin (96–99%, Cohn fraction V), Coomassie Brilliant Blue R, dithiothreitol, Fast Garnet GBC and kanamycin sulphate were from Sigma; Sephadex G-100, sulphopropyl-Sephadex and Blue Dextran were from Pharmacia (G.B.) Ltd., London, U.K. Other chemicals for these techniques were obtained from BDH Chemicals, Poole, Dorset, U.K.

Other reagents

Pure *N*-acetylneuraminic acid had been isolated from boar seminal gel (Boursnell *et al.*, 1970). A solution of *Cholera vibrio* neuraminidase (Behringwerke) was supplied by Hoechst (U.K.) Ltd., Brentford, Middx., U.K., and di-isopropyl phosphorofluoridate was from BDH. Other compounds were from sources specified by Brown *et al.* (1975a,b). Ram semen was collected as described by Brown *et al.* (1975a).

Methods

Processing of ram semen

Spermatozoa were washed with sucrose solutions to free them from seminal plasma and cytoplasmic droplets (Brown *et al.*, 1975a). Heads of spermatozoa (fraction H_w) were isolated as described by Brown & Harrison (1978).

Determination of acrosin activity: units of activity

Activities were measured spectrophotometrically, in terms of hydrolysis at 25°C of Bz-Arg-OEt (Brown *et al.*, 1975a), and are expressed as international units (μmol of substrate hydrolysed/min). Yields of acrosin are given as units derivable from 32ml of semen. This allows direct comparison with an earlier paper (Brown *et al.*, 1975a). Specific activity

and catalytic-centre activity are defined in Table 1. Catalytic-centre molarity of acrosin was determined with 4-methylumbelliferyl *p*-guanidinobenzoate (Brown *et al.*, 1975b).

Column chromatography: experimental conditions and running buffers

Affinity chromatography. Tris/Ca²⁺ buffer contained 50mM-Tris, 20mM-CaCl₂, NaCl as specified, and HCl to give a pH of 8.2 or 8.5. (These buffers slowly deposit CaCO₃ if they are in contact with air, and the pH falls. To obtain reproducible results buffers must be freshly prepared from boiled distilled water.) Imidazole/Ca²⁺ buffer contained 20mM-imidazole, 20mM-CaCl₂ and HCl to pH6.0. NaCl buffer contained 1M-NaCl and HCl to pH2.3.

Gel filtration on Sephadex G-100. The technique of Andrews (1964) was followed. A column (50cm × 2cm²) was calibrated with 1mg each of the proteins serving as molecular-weight standards plus 0.25mg of Blue Dextran as void-volume marker. The flow rate was 12ml/h and 1.2ml fractions were collected. Acrosin was monitored by its reaction with Bz-Arg-OEt, and other proteins by measuring *A*₂₈₀.

Tris/K⁺ buffer was 50mM-Tris/0.5M-KCl and HCl to pH8.0. KCl buffer was 0.5M-KCl and HCl to pH2.6.

Electrophoretic methods

Polyacrylamide disc-gel electrophoresis. We followed procedures described by Brown & Harrison (1978). Briefly, these are (i) electrophoresis at pH4.3 and (ii) staining either for protein (with Coomassie Brilliant Blue) or for acrosin plus proacrosin. The latter technique involves storing the developed electrophoretogram at pH8.2 for 30min at 20°C, to activate proacrosin, then detecting acrosin bands with *N*-α-benzoyl-DL-arginine 2-naphthylamide and Fast Garnet GBC.

SDS/polyacrylamide-gel electrophoresis. The procedure was that of Banker & Cotman (1972) except that (i) the running buffer contained 0.1% SDS, (ii) the gels (120mm × 38mm²) contained 7.1% acrylamide and 0.4% *NN'*-methylenebisacrylamide. Before electrophoresis, acrosin was denatured by a modification of the method of Banker & Cotman (1972). Thus the acrosin was dissolved in the dithiothreitol solution (which caused complete inactivation) and then, after 10min, the SDS solution was added.

Solutions of denatured acrosin received 0.05vol. of glycerol before being applied to the gels. About 10μg of acrosin was applied together with 2.5μg of Bromophenol Blue as front marker. For determinations of the molecular weight of acrosin a mixture of denatured standard proteins (15μg of each protein)

was also placed on the gel. Electrophoresis was run at 9mA/gel for about 3h at room temperature, and then gels were stained with Coomassie Brilliant Blue (Reid & Bielecki, 1968).

Chemical analyses of acrosin

Amino acids. These were determined with a JEOL JLC 5AH automatic amino acid analyser used in conjunction with a JEOL model DK integrator. Three portions of acrosin (each 0.8–0.9mg) were oxidized with performic acid and hydrolysed in 6M-HCl for 24, 48 and 72h. Estimates of amino acid content were made by taking mean values or by extrapolation to zero time of hydrolysis. Tryptophan was estimated in a fourth portion after hydrolysis for 24h in 4M-methanesulphonic acid containing 0.2% of 3-(2-aminoethyl)indole (Liu & Chang, 1971).

Carbohydrate. Total orcinol-reactive carbohydrate was measured by the method of Vasseur (1948) with galactose as standard. Sialic acid, as *N*-acetylneuraminic acid, was determined after acrosin had been treated with neuraminidase. Acrosin (1.0mg) was mixed with 1.1ml of TMM buffer (Bournsnel *et al.*, 1970) and 20μl of neuraminidase. The mixture was stored for 10 days at 4°C, then for 5 days at 20°C. Periodically 0.2ml portions were withdrawn for assay of free *N*-acetylneuraminic acid by the method of Skoza & Mohos (1976).

Ultracentrifugal procedure for measuring molecular weight

Inactivation of acrosin with di-isopropyl phosphorofluoridate. A 0.1M solution of the phosphorofluoridate in propan-2-ol was diluted 45-fold with a buffer containing 50mM-Tris, 50mM-CaCl₂ and HCl to pH8.2. In 0.5ml of this solution was dissolved 3.5mg of acrosin. The mixture was stored for 5h at room temperature and then for 18h at 4°C. Less than 1% of the original activity remained. The mixture was then dialysed exhaustively against the phosphate/saline buffer used for runs in the ultracentrifuge (16mM-Na₂HPO₄, 1.4mM-KH₂PO₄, 0.15M-NaCl, pH7.8). We handled the dangerous phosphorofluoridate in an efficient fume cupboard while wearing disposable polythene gloves.

Determination of the molecular weight of ram acrosin. A Beckman model E analytical ultracentrifuge was set up for meniscus-depletion equilibrium sedimentation (Yphantis, 1964; Chervenka, 1969). Dilutions of the solution of inactivated acrosin were prepared in phosphate/saline: their approximate concentrations were determined from the *A*₂₈₀. These solutions were dialysed against phosphate/saline buffer before being placed in double-sector cells for 'dual runs'. In a dual run, equilibrium was established

by operating the centrifuge at a fixed speed for 20–24 h. After the fringes had been photographed a fresh equilibrium was established at a higher speed and the fringes were photographed again. All runs were at 24.5–25.5°C.

In the calculation of molecular weight

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{2.303 \cdot d \log c}{dr^2}$$

(where c is corrected fringe displacement and r is distance from the centre of rotation at which the displacement was measured) we used 0.728 for the partial specific volume, $\bar{v}$, of acrosin. This was calculated by the method of Cohn & Edsall (1943) by using their values for specific volumes of amino acid residues. We assumed the presence of six sugar residues and used as their mean specific volume the mean of values for hexose and *N*-acetylhexosamine, namely 0.64 (Gibbons, 1972). Values for $d(\log c)/dr^2$ were derived from plots exemplified by Fig. 4 (Yphantis, 1964).

Results

Procedures for the isolation from spermatozoa of homogeneous acrosin

For the final stages of purification the starting materials were two acrosin fractions of similar specific activities: FD and S6. Their relationship is shown in Scheme 1. We retain the original nomenclature of fractions used by Brown *et al.* (1975a).

Standard procedure. The preliminary fractionation, up to the isolation of the stable freeze-dried fraction FD, is described by Brown *et al.* (1975a). Fraction FD was dissolved in Tris/Ca²⁺ buffer (Scheme 1) and applied to a column of adsorbent (I) which had been equilibrated with the same buffer.

Jameson & Elmore (1974) separated bovine α - and β -trypsins on columns of adsorbent (I) by stepwise changes of elution buffers. We found that, when fraction FD was placed on such a column at pH 8.2, acrosin was retained while inactive protein passed through unretarded. Subsequently we removed a major acrosin fraction with imidazole/Ca²⁺ buffer, pH 6.0, followed by a minor one with NaCl buffer, pH 2.3. However, the sharp changes in pH and ionic strength tended to cause discontinuities in the column. Also, although the acrosin in the first fraction was quite stable at pH 6 and at pH 2.3, the acrosin of the second fraction was not. We overcame these difficulties by using an acrosin inhibitor (*p*-aminobenzamidine dissolved in the pH 6 buffer) to elute the second fraction. Thus in the experiment of Fig. 1, 918 units were applied to the column, 715 units were recovered in fraction A and 214 units in fraction B. The presence of 0.25 mM *p*-aminobenzamidine in

Scheme 1. Summary of the steps in the preparation from ram spermatozoa of fraction FD (Brown *et al.*, 1975a) and of fraction S6

→, Original method for isolation of fraction FD; →, modification that constitutes part of the accelerated method leading to fraction S6. 'Clarify' indicates a brief centrifugation to remove traces of an acrosin-free precipitate.

fraction B did not seriously interfere with measurements of acrosin activity since solutions were diluted 500–1000 times in the assay system. At such dilutions the *p*-aminobenzamidine inhibited the acrosin-Bz-Arg-OEt reaction by less than 10%. The high u.v. absorption of the inhibitor prevents measurement of protein at 280 nm. However, when fraction B is eluted with NaCl buffer, pH 2.3, the ratio of activity to A_{280} is close to the ratio for fraction A. No protein remains on the column after fraction B has been eluted, whether this be done with *p*-aminobenz-

amidine at pH 6 or with NaCl, pH 2.3. We therefore conclude that the specific activity of fraction B was similar to that of fraction A.

Accelerated procedure. It is possible to store washed spermatozoa, and denuded spermatozoa (Brown *et al.*, 1975a), in 0.25M-sucrose at -20°C for periods up to 6 months without significantly diminishing the recovery of enzyme units in the derived fraction S3/4 (Scheme 1). For this reason, and also because supplies of ram semen were intermittent, intervals between the collection of semen and the isolation from it of fraction FD varied between 1 and 6 months. In the course of our work we discovered that, despite the good overall recovery of acrosin during the preparation of fraction FD (Brown *et al.*, 1975a), autodigestion of the enzyme was taking place. But initial steps of autodigestion were not accompanied by marked decreases in acrosin activity. To minimize autodigestion we accelerated our standard procedure as follows.

Day 1. Freshly collected semen (not more than 16ml) was centrifuged and the spermatozoa were washed to provide a suspension in 0.25M-sucrose, which was frozen at -20°C (Brown *et al.*, 1975a).

Day 2. After 18 h the frozen suspension was thawed and stored for 0.5 h at 4°C (instead of 2 h at room temperature). The original procedure (Brown *et al.*, 1975a), beginning with denudation, was then followed as far as the production of fraction S3/4. Still kept below 5°C , fraction S3/4 was treated with 0.05 vol. of 1M- CaCl_2 , adjusted to pH 8.5 by cautious addition of 1M-Tris, centrifuged for 10 min at 16000g to remove a fine precipitate, and applied (as S6) to a column of adsorbent (I). This column was run as in Fig. 1, except that Tris buffers were at pH 8.5. Two acrosin peaks were obtained, but the second (fraction B) was very small: as a rule it contained less than 6% of the acrosin units that had been applied to the column (Hartree, 1977). Fraction A was dialysed for 4 h against 1mM-HCl, then overnight against fresh 1mM-HCl.

Day 3. The enzyme fraction was dialysed for 2.5 h against glass-distilled water and freeze-dried.

Freeze-dried fraction A, and the similarly dialysed and freeze-dried fraction B, are designated β -acrosin and ψ -acrosin respectively. [The term α -acrosin has been used for an acrosin species which has a lower electrophoretic mobility than β -acrosin (Brown & Harrison, 1978) and which appears to be a precursor of β -acrosin.] When stored at 5°C over silica gel β -acrosin is very stable. The yields of these fractions and the activities of β -acrosin are given in Table 1.

The value 13.9 was obtained for $A_{280}^{1\text{cm}}$ of a 1.00% (w/v) solution, in 1mM-HCl, of β -acrosin that had been dried to constant weight *in vacuo* over P_2O_5 .

Fig. 1. Purification of acrosin FD by affinity chromatography on a column of adsorbent (I)

The protein in 33 mg of fraction FD that was soluble at pH 8.2 (Scheme 1) was placed on a column $13\text{ cm} \times 0.8\text{ cm}^2$. Eluting solutions were (i) Tris/ Ca^{2+} buffer, pH 8.2; (ii) Tris/ Ca^{2+} buffer containing 0.5M-NaCl, (iii) imidazole/ Ca^{2+} buffer, pH 6.0; (iv) imidazole/ Ca^{2+} buffer containing 0.25 mM-*p*-aminobenzamidine. [Elution with buffer (ii) sometimes eluted additional acrosin-free protein (Hartree, 1977).] Flow rate was 28 ml/h; volume of eluent/tube was 4.8 ml; $t = 5^{\circ}\text{C}$. Protein was monitored as $A_{280}^{1\text{cm}}$. Acrosin activity was measured immediately after tube fractions had been collected. Then, without delay, appropriately pooled solutions were adjusted to pH 2.3 with HCl and dialysed against 1mM-HCl followed by glass-distilled water. The products were freeze-dried and were stored at 5°C over silica gel. ●, Acrosin; ----, A_{280} .

Activation of proacrosin

Transformation of proacrosin to acrosin during the isolation of acrosin from spermatozoa. This process was followed by correlating the acrosin activities of fractions obtained during the purification of acrosin with the band patterns resulting from disc-gel electrophoresis of the fractions (Fig. 2).

When spermatozoa from freshly collected ram semen were washed with sucrose and, instead of being denuded, were immediately extracted at pH 2.7 (Brown *et al.*, 1975a) the acrosin activity of the acid extract was very low. Electrophoresis of the extract, followed by staining for acrosin plus proacrosin yielded a strong band with R_F 0.45 and a very faint, faster-moving band (Fig. 2, gel 1). An acid extract of frozen-thawed spermatozoa had much higher acrosin activity and showed, in addition to the original band, two faster-moving weaker bands (Fig. 2, gel 2).

Similar treatments of samples taken at successive steps in the purification of acrosin by the accelerated method demonstrated further activation and also progressive transformation of the original main protein component into β -acrosin, which is represented by a single band with R_F 0.60 (Fig. 2, gels 2–5). In freshly collected ram spermatozoa, therefore, acrosin is present very largely as proacrosin. Thus if, in the course of staining electrophoretogram 1 (Fig. 2), storage of the gel at pH 8 is omitted, a weaker main band is obtained.

Fig. 2. Diagrams of disc-gel electrophoretograms, also acrosin activities, obtained with six fractions isolated during the purification of acrosin

1, Acid extract of freshly washed spermatozoa; 2, acid extract of frozen washed spermatozoa; 3, acid extract of freshly denuded spermatozoa; 4, fraction S6 (Scheme 1); 5, 20 μ g of β -acrosin; 6, 20 μ g of ψ -acrosin. The material applied to a gel as an acid extract corresponded to about 4×10^6 spermatozoa. Gels 1–5 were run with fractions obtained by the accelerated procedure; gel 6 was run with ψ -acrosin obtained as in Fig. 1. All gels were stained for acrosin plus proacrosin. Acrosin activities are units in fractions derived from 32 ml of ram semen (approx. 10^{11} spermatozoa). Since ψ -acrosin (gel 6) is unstable no value for units is given.

Since activation of proacrosin is complete in terms of enzyme units at the stage S1/2, presumably all bands except the slowest are active forms of acrosin.

When electrophoretograms of β -acrosin and of fresh ψ -acrosin were stained for protein they showed bands only in the positions where acrosin was detectable.

ψ -Acrosin contains three components with acrosin activity. Attempts to separate these on columns of adsorbent (I) by stepwise changes of pH and ionic strength have been unsuccessful. Each change in buffer composition gave rise to a very small peak, and no peak represented a single protein component. We passed ψ -acrosin through a column of sulphopropyl-Sephadex, using NaCl gradient in buffer (iv) (see legend to Fig. 1). Two acrosin peaks emerged, but both consisted of mixed acrosin components. We regard ψ -acrosin as mixed autodigestion products of β -acrosin.

Effect of Bz-Arg-OEt on the activation of proacrosin. An acid extract of freshly washed spermatozoa had very low acrosin activity (Fig. 2). This was not due to the presence of the intra-acrosomal inhibitor of acrosin, because in the assay conditions (addition of the acid extract to a cuvette containing buffered Bz-Arg-OEt) enzyme and inhibitor remain dissociated (Brown *et al.*, 1975a; Brown & Hartree, 1976). The recorder trace from which the activity of this extract was calculated remained linear for 2 min, i.e. there was no progressive activation of proacrosin. In the assay of acrosin activity the molar ratio substrate/(enzyme + proenzyme) is very high, about 10000. It therefore appeared important to look for effects of Bz-Arg-OEt on the activation reaction.

Acid extracts of denuded spermatozoa contain much less inhibitor than acid extracts of fresh spermatozoa, but the former already show enhanced acrosin activity (Fig. 2). So for the experiments of Fig. 3 our source of proacrosin was the sperm-head fraction H_w (Brown & Harrison, 1978). On account of damage to

Table 1. Yields and activities of β - and ψ -acrosins obtained by two procedures for the purification of ram acrosin. Yields are mean values based on four fractionations by the standard procedure and four by the accelerated procedure. During standard procedures quantities of acrosin applied to a column of adsorbent (I) were from 16–32 ml of semen. During accelerated procedures quantities were from 8–16 ml of semen. Yields have been recalculated to correspond to 32 ml of semen. In assay conditions the enzyme is 90% saturated with substrate. Estimates of yields and activities related to V_{max} can therefore be obtained by increasing quoted values by 10%.

Isolation procedure	Yields of acrosins from 32 ml of semen (units)		Catalytic activity of β -acrosin at 25°C	
	β -Acrosin	ψ -Acrosin	Sp. activity (units/mg of freeze-dried protein)	Catalytic-centre activity (mol of Bz-Arg-OEt hydrolysed/s by 1 mol of acrosin)
Standard	705	220	138	137
Accelerated	1600	105	142	138*

* The corresponding value for boar acrosin is 148 (Schleuning *et al.*, 1975a).

Fig. 3. Effect of Bz-Arg-OEt on the activation of proacrosin in a neutralized acid extract of sperm heads
The acid extract was diluted with Tris/Ca²⁺ buffer, pH 8.2 (Fig. 1), containing different concentrations of Bz-Arg-OEt. These solutions were stored at 25°C and 0.2 ml samples were withdrawn periodically for assay of acrosin with Bz-Arg-OEt. The stored solutions contained the following concentrations of Bz-Arg-OEt: ○, 0; □, 0.2 mM; △, 1.0 mM; ▲, 2.5 mM. ■, Zero-time acrosin activity determined by adding acid extract directly to buffered Bz-Arg-OEt in a spectrophotometer cuvette; ■, position of overlapping experimental points on all curves.

the acrosomes the heads in this fraction are free from the acrosomal inhibitor. Also, they yield acid extracts with very low acrosin activity.
A suspension of sperm heads was used to prepare an acid extract (Brown *et al.*, 1975a), 1 ml of which contained soluble material from about 7.5×10^8 heads. As a control solution, 0.4 ml of this extract was diluted to 2 ml with Tris/CaCl₂/HCl buffer, pH 8.2, the final concentrations of Tris and CaCl₂ being 50 mM and 0.2 M respectively. Three experimental solutions were prepared in the same way but with Bz-Arg-OEt present. The four solutions were stored at 25°C and portions were periodically assayed for acrosin activity. (Had inhibitor been present it would have associated with the acrosin during storage.) When samples were transferred to cuvettes containing buffered substrate, concentrations of substrate rose above the standard 1 mM. However, this would have had insignificant effects on reaction rates (see the legend to Table 1).

Table 2. Amino acid analysis of β -acrosin

	Content (μ mol/mg of acrosin)	Molar ratio		
		Calc.	Nearest integer	Values for boar acrosin*
Lys	0.338	15.9	16	19
Arg	0.526	24.8	25	23
His	0.188	8.9	9	6
Asp	0.504	23.8	24	27
Glu	0.615	29.0	29	29
Thr	0.409	19.3	19	20
Ser	0.524	24.7	25	23
Pro	0.767	36.2	36	31
Met	0.086	4.1	4	7
Gly	0.661	31.2	31	38
Ala	0.536	25.3	25	22
Val	0.546	25.8	26	22
Ile	0.386	18.2	18	17
Leu	0.441	20.8	21	20
Tyr	0.217	10.2	10	8
Phe	0.222	10.4	10	9
Cys	0.252	11.9	12	12
Trp	0.066	3.1	3	3

Sum of residue weights: 37 200

* From Schleuning *et al.* (1975b).

The initial lag period in the activation of proacrosin during storage increased with the concentration of Bz-Arg-OEt (Fig. 3). However, even with the control solution there was an interval of about 10 min between the making up of that solution and the onset of activation. In Fig. 3 some experimental points represent assays that were carried out at times of rapid activation. Even so, rates of hydrolysis of Bz-Arg-OEt by the samples remained linear. This was due in part to the short time required for the acrosin assay, and presumably also to inhibition of activation by the substrate in the cuvette.
Calculations based upon (a) the relationship between units of activity and enzyme molarity, and (b) the kinetic constants of the acrosin/Bz-Arg-OEt reaction (Brown *et al.*, 1975a) indicate that development of maximum, and parallel, activation rates in Fig. 3 coincides with complete hydrolysis of substrate in the stored solutions.

Amino acids and carbohydrate in β -acrosin

Table 2 gives the amino acid composition of β -acrosin together with published values for boar acrosin.
Total carbohydrate was 2.87%, or 5.9 'galactose' residues/molecule. If the acrosin had contained one N-acetylneuraminy residue/molecule of enzyme, a value of about 0.43 would have been obtained for A_{531}^{cm} in the assay of liberated N-acetylneuraminic acid. However, experimental A values were indis-

tinguishable from those obtained in blank (minus neuraminidase) runs. The neuraminidase was checked with Cowper's-gland mucin (Boursnell *et al.*, 1970) and shown to be very active. Schleuning *et al.* (1975a) found that the electrophoretic mobility of purified boar acrosin changed considerably after it had been treated with neuraminidase, but they gave no value for sialic acid content.

Estimates of the molecular weight of acrosin

SDS/polyacrylamide disc-gel electrophoresis of β - and ψ -acrosins. When β -acrosin had been denatured by the customary method (dissolution in SDS, followed 10 min later by addition of dithiothreitol) the stained electrophoretogram showed several protein bands. There was one intense band plus at least four much fainter and more rapidly moving bands. However, when we used our method for denaturation, the same intense band appeared but no others.

β -Acrosin was run in conjunction with the following proteins: cytochrome *c* (12000), myoglobin (17800), chymotrypsinogen (25000), ovalbumin (45000) and bovine serum albumin (67000). The customary plot of $\log(\text{molecular weight})$ against R_F was a straight line for the standard proteins. Interpolation of the R_F for β -acrosin gave 38000 for the molecular weight.

Electrophoresis of denatured ψ -acrosin yielded an intense band that was more diffuse than that of β -acrosin. However, it represented a molecular weight that was indistinguishable from that of β -acrosin. There were also at least three faint bands corresponding to mol.wts. between 27000 and 34000.

Gel chromatography of β - and ψ -acrosins. These two forms of the enzyme were each run at two pH values on a column of Sephadex G-100. The column was calibrated with the following standard proteins: soya-bean trypsin inhibitor (22000), chymotrypsinogen, ovalbumin and serum albumin. The semi-logarithmic plots of the results obtained with the standards were linear. Molecular weights of the acrosins, as read off from these plots, were as follows: β -acrosin, 45200 at pH2.6 and 44500 at pH8.0; ψ -acrosin, 42600 at pH2.6 and 43200 at pH8.0. Values for ψ -acrosin are presumably weighted means for the components that are detectable by SDS/polyacrylamide disc-gel electrophoresis.

Meniscus-depletion equilibrium sedimentation of β -acrosin. Attempts to determine the molecular weight of unmodified β -acrosin were unsuccessful. Even at a pH value as low as 2.4 the plots of $\log c$ against r^2 (as in Fig. 4) were curved, i.e. the acrosin had been degraded to at least two fragments during the time necessary for establishment of equilibrium.

Fig. 4. Meniscus-depletion equilibrium sedimentation of β -acrosin

A representative plot of $\log c$ against r^2 , where c = corrected fringe displacement (μm) and r = distance (cm) from the centre of rotation at which the displacement was measured.

Schleuning *et al.* (1975b) obtained linear plots when studying equilibrium sedimentation of boar acrosin that had been inactivated with di-isopropyl phosphorofluoridate. Our experience has been similar. The measured fringe displacements at different r values, from all runs with inactivated β -acrosin, gave rise to linear plots (e.g. Fig. 4). Thus this acrosin fraction is homogeneous in terms of molecular weight.

Three dialysed solutions of inactivated β -acrosin were prepared in phosphate/saline buffer (see the Methods section). They contained 0.2, 0.4 and 0.6% of protein. Each solution was used for a dual run. The fringe photographs thus provided information for six plots of $\log c$ against r^2 : three from equilibria at 24000 rev./min and three at 28000 rev./min. The three calculated values of molecular weight for each rotor speed were independent of protein concentration: they varied by less than 1.5% from the mean. The two mean-of-three values were 40000 and 39400. A replicate experiment yielded the values 39400 and 39600. The mean of these four results gives 39600 for the molecular weight of β -acrosin.

Table 3. *Stability of stored solutions of β -acrosin*

All solutions contained 150 μ g of enzyme/ml. Dulbecco solution (Whittingham, 1971) contained 4mg of serum albumin/ml, and Tyrode solution (Paul, 1960) 1 mg of serum albumin/ml. Kanamycin sulphate (50 μ g/ml) was added to both solutions.

Solvent for acrosin	pH	Storage temperature (°C)	Initial acrosin activity (unit/20 μ l)	Activity remaining (%)		
				After 3 h	After 22 h	After 50 h
Dulbecco solution	7.4	25	0.172	106	99	94
		37		92	52	41
Tyrode solution	7.4	25	0.194	96	64	42
		37		40	17	0
1 mM-HCl	2.4	25	0.211	96	92	92
20 mM-Imidazole/HCl						
+ 80 mM-NaCl	6.0	25	0.192	91	44	32
+ 20 mM-CaCl ₂	6.0	25	0.187	104	99	97
0.1 M-Tris/HCl	8.0	25	0.171	93	79	63
+ 20 mM-CaCl ₂	8.0	25	0.185	105	88	78
+ 100 mM-CaCl ₂	8.0	25	0.189	103	81	69

Changes in the activities of stored acrosin solutions

Portions of a solution of β -acrosin in 1 mM-HCl (1.5 mg/ml) were diluted with 9 vol. of each of the solutions listed in Table 3. Mixtures were stored at 25 or 37°C, and 20 μ l portions were taken periodically for assays with Bz-Arg-OEt. The essential results were as follows. (1) Enzyme activity persisted longer in Dulbecco solution than in Tyrode solution. (2) Activation of freshly prepared acrosin solution by Ca²⁺ at pH8 was less marked than activation of the impure fraction FD (Brown *et al.*, 1975a). There was no activation by Ca²⁺ at pH6. (3) Stabilization of acrosin by Ca²⁺ was more complete at pH6 than at pH8: the enzyme was as stable in imidazole/Ca²⁺ buffer, pH6, as it was in 1 mM-HCl.

Dulbecco and Tyrode solutions, with added albumin and antibiotic, were included in these tests since they are typical media for experiments with mammalian eggs *in vitro*, including study of the effects of acrosin on eggs (Hartree, 1977).

It must be emphasized that these experiments do not provide measures of the stability of β -acrosin. The transformation of β -acrosin into ψ -acrosin occurs in solution, especially at pH8.

'Hydrophobic affinity' chromatography of ram acrosin and bovine trypsin

Acrosin is bound to the inner acrosomal membrane of the sperm head (Hartree, 1977). The surfaces of isolated membrane protein molecules are known to possess apolar (hydrophobic) regions (Gittler, 1972); also, compound (I) has an extended apolar, aromatic-aliphatic chain. Thus it seemed possible that binding of acrosin to adsorbent (I) is governed, not only by the affinity of the amidine group for the active-site

Fig. 5. *Hydrophobic affinity chromatography of acrosin on a column of adsorbent (II)*

Column dimensions, flow rate and volume of eluent/tube were as in Fig. 1. Acrosin FD (248 units) was dissolved in Tris/Ca²⁺ buffer containing 3 M-NaCl (v). The column was developed with buffer (v) followed by buffer (i) of Fig. 1.

region of this proteinase, but also by less specific hydrophobic interactions (Hofstee, 1974).

We have used adsorbent (II) to test for hydrophobic bonding between acrosin and the hydrophobic region that is common to compounds (I) and (II). On the basis of work by Hofstee (1975), a column of adsorbent (II) was equilibrated with Tris/Ca²⁺ buffer, pH8.2, containing 3 M-NaCl (buffer v, Fig. 5). When

acrosin FD was placed on this column and washed through with more buffer (v), a peak of inactive protein was eluted. Change to NaCl-free Tris/Ca²⁺ buffer then released all acrosin as a second sharp peak. The recovered acrosin solution was dialysed against buffer (v) and re-run on the same column but with a linear NaCl gradient (3M to 1M) in Tris/Ca²⁺ buffer. Acrosin emerged as a single wide peak the summit of which was at 2.2M-NaCl. This material was transferred to a column of adsorbent (I), washed first with buffer (v) and then with the buffer sequence of Fig. 1. No fraction A was obtained, but all the proteinase emerged as fraction B. The overall recovery of acrosin activity was less than 30%. Since compound (II) is not an inhibitor of acrosin, autodigestion at pH 8.2 could presumably occur whether or not the enzyme was bound to adsorbent (II). High concentrations of salt suppress ionization of the secondary amino groups that are formed when compounds (I) and (II) react with activated Sepharose; they also enhance hydrophobic interactions (Hofstee, 1975). Thus in the presence of 3M-NaCl the binding properties of adsorbent (II) are predominantly hydrophobic.

Trypsin could not be induced to bind to adsorbent (II). Its passage through the adsorbent was unretarded at all concentrations of NaCl up to 3M.

Discussion

Formation of β -acrosin from precursors and its subsequent autolysis

When freshly collected spermatozoa are extracted at low pH values most of the acrosin is solubilized in the form of the inactive proacrosin. At pH 8 soluble proacrosin undergoes activation to give multiple, and mostly transient forms of acrosin. These phenomena have been observed with spermatozoa of rabbit, hamster, bull, ram, boar and man (Fritz *et al.*, 1974; Meizel & Mukerji, 1975, 1976; Schleuning *et al.*, 1976a,b; Shams Borhan *et al.*, 1976; Polakoski & Parrish, 1977; also the present paper). When one intermediate is more stable than the others it becomes possible to isolate a pure form of acrosin, e.g. ram β -acrosin and the boar acrosin of Schleuning *et al.* (1975a). The latter authors noted that if boar spermatozoa had been stored, additional acrosin components were present in acid extracts. When we purified ram acrosin from denuded spermatozoa that had been stored at -20°C for at least 1 month, about 25% of the acrosin units were recovered as ψ -acrosin, which is a mixture of unstable forms. It is not clear from these results whether ψ -acrosin is formed from β -acrosin or from precursors of β -acrosin. Evidence in favour of the former view is that when β -acrosin was adsorbed on, and eluted from, adsorbent (II) it was converted into a form that behaved as ψ -acrosin during chromatography on adsorbent (I).

A partial self-digestion of acrosin occurred during the 'customary method' of denaturation (i.e. during 10min contact of acrosin with SDS: see under 'Electrophoretic methods'). This is noteworthy because the hydrolysis of Bz-Arg-OEt by acrosin is abolished within 10s by as little as 0.1% SDS.

Variations in the abilities of adsorbents (I) and (II) to bind acrosin

We have described in more than customary detail our standard method for preparing adsorbent (I). This is because the properties of the product can vary if the method for activation of Sepharose is not followed precisely. Conditions for the coupling reaction are less critical and we have retained the procedure of Jameson & Elmore (1974). By using lower concentrations of CNBr or by activating Sepharose at 0°C we obtained adsorbents with lower binding capacities for acrosin and also unacceptably low binding affinities. More vigorous activation (higher concentrations of CNBr, increases in temperature and duration of activation) did not result in appreciably higher capacities, but acrosin became more firmly bound. An additional disadvantage of vigorous activation was an apparent inhomogeneity of binding sites. Thus even with an apparently pure acrosin there might be a slow and irregular 'bleed' before the major peak emerged, or an irregular trail of enzyme after the peak had been collected. Also, peaks tended to be less sharp and less symmetrical than those obtained with the standard adsorbent (I).

We believe that these non-ideal samples of adsorbent (I) were exhibiting properties other than what can be conveniently described as primary (affinity) binding, i.e. chemically specific binding of the enzyme-inhibitor type. In fact, affinity adsorbents that show only such primary binding properties are unattainable (O'Carra *et al.*, 1974). Coupling CNBr-activated Sepharose to a primary amine gives rise to a positively charged adsorbent (Nishikawa & Bailon, 1975), and even purified agarose carries sufficient negative charge to bind basic proteins weakly (Hofstee, 1973). When a Sepharose-bound ligand contains non-polar regions there is possibility of hydrophobic bonding between the immobilized ligand and a protein with hydrophobic surface regions (Hofstee, 1973, 1974, 1975; Ochoa, 1978). Experiments with adsorbent (II) showed that the aliphatic-aromatic diether structure common to compounds (I) and (II) can bind acrosin at high ionic strength, i.e. in conditions that enhance hydrophobic bonding but suppress ionic interactions (Hofstee, 1975). Ionic interactions are unlikely to be important among the forces that bind acrosin to adsorbents (I) and (II). Acrosin is positively charged in the experimental conditions as are also both adsorbents.

Amidine groups contribute additional positive charge. Although primary binding can provide a high degree of specificity, the bonding strength may be determined largely by supplementary hydrophobic contacts (Chothia & Janin, 1975; Anttinen *et al.*, 1977). Unlike primary sites, immobilized hydrophobic sites are customarily heterogeneous (Hofstee, 1974).

Increasingly vigorous activation, for the preparation of adsorbent (I), presumably leads to an increase in the surface density of covalently bound ligands. When the mean distance between bound ligands becomes less than the distance between centres of close-packed bound acrosin molecules there can be no increase in the primary binding capacity of the adsorbent. However, there will be increasing probability of additional hydrophobic interactions. Our attempts to separate the components of ψ -acrosin on adsorbent (I) by multistage changes of buffer composition gave rise to numerous small peaks but to no resolution of ψ -acrosin. This is consistent with hydrophobic interactions exhibiting randomly variable affinity. Our empirically selected (standard) form of adsorbent (I) seems to have the optimum combination of primary binding properties and hydrophobic characteristics.

Nishikawa & Bailon (1975) found that α -chymotrypsin and β -galactosidase exhibit especially high affinity for immobilized hydrophobic ligands with aromatic rings. We were unaware of this work when we selected for trial, as ligand, a benzamidine with two aromatic rings. Our choice was influenced by the following facts. (1) This benzamidine is a powerful inhibitor of trypsin (Baker & Erickson, 1968). (2) It has a built-in spacer arm. (3) As an immobilized ligand it was effective in the separation of α -trypsin from β -trypsin (Jameson & Elmore, 1974). Our work with adsorbents (I) and (II) suggests that it may be worthwhile to make a preliminary study of the effects of different activation procedures on the properties of an affinity adsorbent. If a selected procedure is then followed rigorously it is likely that all batches of the adsorbent will have the required properties.

Molecular weights of acrosin and proacrosin

Meizel & Mukerji (1975) reported values of 73 000 and 38 000 respectively for the molecular weights of rabbit proacrosin and completely activated acrosin. An intermediate in the conversion was an active acrosin with mol.wt. 73 000 (Mukerji & Meizel, 1975). These results suggest that activation of rabbit proacrosin involves the splitting of a dimeric protein and the possible formation of two molecules of active proteinase (Mukerji & Meizel, 1975; McRorie *et al.*, 1976). However, the existence of proacrosin as a dimer has not been confirmed for other species. Values given

by other workers for reputedly pure rabbit acrosin range from 27 000 to 59 000 (Meizel & Mukerji, 1975). A similarly wide range of values is on record for human acrosin (Meizel & Mukerji, 1975; Schleuning *et al.*, 1976b; Tobias & Schumacher, 1977).

Results obtained, by means of SDS/polyacrylamide-gel electrophoresis, for boar acrosin suggest that the first stable activation product has a mol.wt. of 38 000 and that this, in turn, is degraded to active products with mol.wts. between 30 000 and 34 000 (Polakoski *et al.*, 1973; Schleuning & Fritz, 1974). However, different methods for obtaining molecular weights do not give results in good agreement. Recorded values for the molecular weight of the one form of boar acrosin that has been purified to homogeneity are 56 000 by gel filtration (Fink *et al.*, 1972), 38 000 by SDS/polyacrylamide-gel electrophoresis (Schleuning & Fritz, 1974), and 41 000 by equilibrium centrifugation (Schleuning *et al.*, 1975b). Values for ram β -acrosin, obtained by the same methods, are respectively 44 000–45 000, 38 000 and 39 600. The high values obtained by the first method may be due to the presence of carbohydrate; thus the boar acrosin was reported to contain 10% of carbohydrate and the ram acrosin appears to contain about 3%. It is noteworthy that Gilboa *et al.* (1973) obtained almost identical values for the molecular weight of human acrosin by gel filtration and by SDS/polyacrylamide-gel electrophoresis, but they did not analyse their product for carbohydrate.

The main uncertainty in the equilibrium-centrifugation method is the value of $\bar{v}$. Experimentally determined values do not as a rule agree with calculated values (Cohn & Edsall, 1943), and it requires a decrease of only 1.5% in our value for $\bar{v}$ (0.728) to decrease the mol.wt. to about 38 000. A fourth value for the molecular weight of β -acrosin can be obtained from the sum of amino acid residue weights (Table 2). Addition of six hexose residues gives the value 38 100. We therefore suggest 38 000 as a provisional molecular weight for β -acrosin.

Purification of the most stable soluble forms of boar and ram acrosin has established not only that they have similar catalytic-centre activities and molecular weights, but also that their amino acid compositions are comparable (Tables 1 and 2). Thus if one takes into account the sums of (1) acidic residues, (2) basic residues, (3) residues with apolar side chains, and (4) serine and threonine, the similarity is quite marked. On the other hand a published amino acid composition of a highly active rabbit acrosin (Stambaugh & Smith, 1974) resembles that of human trypsin, but it is quite different from that of ram acrosin. The presence of sialic acid in boar acrosin but not in ram acrosin may have no significance. One cannot rule out the possibility that terminal sialic acid residues were detached during purification of the latter.

The value 13.9 for $A_{280}^{1\%}$ of β -acrosin may be an underestimate. When a few milligrams of a protein are isolated by freeze-drying, contamination from water, reagents and polysaccharide-based adsorbents cannot be entirely avoided. However, the value is comparable with those of 15.8 for trypsin (Foucault *et al.*, 1974) and 13.9 for trypsinogen (Vincent & Lazdunski, 1976). On the basis of a biuret assay of protein, Schleuning *et al.* (1975a) suggest 16.1 for their pure boar acrosin. However, correction for the estimated 10% of carbohydrate would give a lower value.

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