Characterization of a high-molecular-weight form of human acrosin

Comparison with human pancreatic trypsin

Robert A. ANDERSON, Jr.*† Stan A. BEYLER,†‡ Stephen R. MACK† and Lourens J. D. ZANEVELD†§
Departments of †Physiology and Biophysics, and §Obstetrics and Gynecology, University of Illinois at the
Medical Center, P.O. Box 6998, Chicago, IL 60680, U.S.A.

(Received 25 March 1981/Accepted 1 July 1981)

A high-molecular-weight form of acrosin (α -acrosin, EC 3.4.21.10) was extracted from spermatozoa obtained from frozen semen and purified over 300-fold. Purification was effected by sequential use of Sephadex G-150, CM-cellulose and DEAE-cellulose chromatography. Properties of human acrosin were compared with those of human pancreatic trypsin. The molecular weight (M_r) of acrosin (70 000) was greater than that of trypsin (M_r 21 000). Isoelectric points for acrosin ($pI = 9.0$) and trypsin ($pI = 8.2$) were also different. α -N-Benzoyl-L-arginine ethyl ester was hydrolysed 50% more rapidly by acrosin than by trypsin. Acrosin had similar k_{cat} values for the hydrolysis of esters with different acylating groups (i.e. benzoyl-L-arginine and *p*-tosyl-L-arginine esters). In contrast, trypsin had dissimilar k_{cat} values for the hydrolysis of esters with different acylating groups. Kinetic data argue against deacylation as the rate-limiting step in ester hydrolysis by acrosin. Acrosin was less sensitive than trypsin to inhibition by 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'), di-isopropyl fluorophosphate and soya-bean trypsin inhibitor. D-Fructose and D-arabinose inhibited acrosin, but had no effect on trypsin. The data indicate that definite differences exist between human acrosin and trypsin.

Since the isolation of acrosin (EC 3.4.21.10) from rabbit spermatozoa (Stambaugh & Buckley, 1968, 1970; Polakoski *et al.*, 1972; Zaneveld *et al.*, 1972), great attention has been given to acrosin with respect to its possible role in spermatozoal penetration of the ovum. Acrosin is present in mammalian spermatozoa (Morton, 1977), and has been identified as a serine proteinase with substrate specificity and inhibitor sensitivity similar to that of trypsin (Schleuning & Fritz, 1976). Inhibition of fertilization *in vitro* (Stambaugh & Buckley, 1971; Bhattacharayya *et al.*, 1979) and *in vivo* (Zaneveld

et al., 1970) by acrosin inhibitors supports the importance of acrosin in fertilization.

Acrosin has been partially characterized from several mammalian species, including rabbit (Zaneveld *et al.*, 1972; Stambaugh & Smith, 1974), ram (Morton, 1977), boar (Fink *et al.*, 1972; Zaneveld *et al.*, 1973), bull (Multimaki & Niemi, 1972; Garner & Cullison, 1974), mouse (Gaddum & Blandau, 1970; Fink & Wendt, 1978) and man (Fritz *et al.*, 1972; Gilboa *et al.*, 1973; Bhattacharyya *et al.*, 1979).

Preliminary work by Gilboa *et al.* (1973) indicated that human acrosin may differ from that of other species. A survey of the literature (Morton, 1977) suggested that acrosin may be species specific, indicating the need for the characterization of human acrosin, so that its role in human fertilization might be better understood.

Most acrosin exists as its zymogen, proacrosin (Meizel & Mukerji, 1975; Polakoski & Zaneveld, 1976). When proacrosin is activated, a high-molecular-weight form of acrosin appears, followed in time by the appearance of low-molecular-weight forms (Schleuning *et al.*, 1976). The heterogeneity of acrosin formed from proacrosin activation renders

* To whom requests for reprints should be addressed.

‡ Present address: Department of Biology, Northwestern University, Evanston, IL 60201, U.S.A.

Abbreviations used: Bz-Arg-OEt, α -N-benzoyl-L-arginine ethyl ester; Bz-Arg-OMe, α -N-benzoyl-L-arginine methyl ester; Bz-DL-Arg-Nan, α -N-benzoyl-DL-arginine *p*-nitroanilide; Bz-DL-Arg-NNap, α -N-benzoyl-DL-arginine β -naphthylamide; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; iPr₂P-F, di-isopropyl fluorophosphate; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; SDS, sodium dodecyl sulphate; SP-Sephadex, sulphopropyl-Sephadex.

an accurate characterization of acrosin difficult. The higher-molecular-weight forms are unstable and undergo transformation to lower-molecular-weight forms (R. A. Anderson & S. A. Beyler, unpublished work).

Studies by our laboratory (Goodpasture *et al.*, 1981) indicate that proacrosin is unstable to freezing. After freezing of either whole semen or spermatozoal extracts, proacrosin is inactivated, leaving behind a stable high-molecular-weight form of acrosin (α -acrosin). The objective of the present work was to isolate and characterize human α -acrosin from frozen semen.

Although acrosin isolated from several species apparently has properties similar to those of bovine pancreatic trypsin (Schleuning & Fritz, 1976; Morton, 1977), no comparison has been made between human acrosin and human pancreatic trypsin. We have compared properties of human α -acrosin and pancreatic trypsin (EC 3.4.21.4).

Experimental

All reagents required for polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Bz-DL-Arg-Nan was a product of Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Ac-Tyr-OEt was purchased from Cyclo Chemical Corp. (Los Angeles, CA, U.S.A.). The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.): all other synthetic substrates, Tos-Lys-CH₂Cl and trypsin inhibitor from soya bean. [³H]iPr₂P-F {di-[1-³H(n)]isopropyl fluorophosphate; sp. radioactivity 0.9 Ci/mmol} was obtained from New England Nuclear (Boston, MA, U.S.A.). Non-radiolabelled iPr₂P-F was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Fluorescamine was a product of Roche Diagnostics (Nutley, NJ, U.S.A.). Protein standards for molecular-weight estimation were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Other materials and reagents were of the highest quality commercially available.

Enzyme assays

Activities of human trypsin and acrosin were assayed spectrophotometrically at 253 nm by following the hydrolysis of Bz-Arg-OEt (Schwert & Takenaka, 1955) at 25°C. Reactions contained 0.5 mM-Bz-Arg-OEt, 50 mM-Tris/HCl, pH 8.0, and 0.2 μ g–0.5 mg of enzyme protein, in a volume of 1.0 ml. A change in absorbance of 1.15 corresponded to the hydrolysis of 1.0 μ mol of substrate (Schleuning & Fritz, 1976). Results were expressed as μ mol of Bz-Arg-OEt hydrolysed/min per mg of protein.

Measurement of proacrosin

The proacrosin content of freshly ejaculated or frozen spermatozoa was measured by a modification of the method of Zahler & Polakoski (1977). Semen was made 50 mM in benzamidine hydrochloride before separation of the spermatozoa by centrifugation (1200 g, 15 min; r_{av} = 10.8 cm). All reagents used for proacrosin isolation contained 50 mM-benzamidine hydrochloride. The spermatozoa were resuspended in 0.1 M-sodium phosphate, pH 7.5 (one-half original semen volume) and layered over 1.0 M-sucrose and centrifuged at 6000 g for 30 min. The pellet was suspended in 66 mM-acetic acid (one-quarter original semen volume), adjusted to pH 2.8 with 1.0 M-HCl and extracted for 18 h at 4°C (with stirring). The suspension was centrifuged (13 000 g, 15 min) and the supernatant dialysed against two changes of 1000 vol. of 1 mM-HCl (no benzamidine hydrochloride added). Proacrosin from the dialysed extract was measured as follows: equal volumes of extract and 50 mM-Tris/HCl, pH 8.0, were added to plastic tubes and incubated at 25°C for 5–120 min. Samples were adjusted to pH 2.8 (with 1.0 M-HCl) and assayed for acrosin activity. Proacrosin content was taken as the difference in acrosin activity before and after incubation.

Purification of human acrosin

Frozen semen was provided by Dr. S. Leto (Washington Infertility Clinic, Washington, DC, U.S.A.) or by Dr. A. D. Morris (Midwest Population Center, Chicago, IL, U.S.A.). Semen was frozen and shipped in liquid nitrogen and stored at –70°C. Plasticware was used to minimize enzyme loss by adsorption to glass (Meizel & Huang-Yang, 1973; Schleuning & Fritz, 1976).

Extraction of acrosin

Semen (250 ml) was thawed at 4°C (average sperm count = 60×10^6 /ml) and filtered through six layers of cheesecloth. All procedures were conducted at 4°C. Acrosin extraction was similar to proacrosin extraction, though benzamidine was omitted from all solutions. No difference resulted in recovered acrosin when extraction time varied from 4 to 18 h. Extraction was therefore carried out for 4 h. The supernatant after centrifugation (13 000 g, 15 min) was kept.

Sephadex chromatography

The extracted acrosin was concentrated [Amicon PM 10 membrane pre-treated with 2% bovine serum albumin, under nitrogen at 380 kPa (55 lb/in²) and applied to a Sephadex G-150 column (2.5 cm $\times$ 63 cm; flow rate 6.1 ml $\cdot$ cm⁻² $\cdot$ h⁻¹). The column was eluted with 55 mM-acetic acid/0.1 M-NaCl; 3.0 ml fractions were collected.

CM-cellulose chromatography

Acrosin eluted from the Sephadex column was concentrated (Amicon PM 10 membrane) and made 10mM with respect to sodium citrate. The pH was adjusted to 5.5 (1M-NaOH) and applied to a CM-cellulose column (1.0cm $\times$ 10.0cm) previously equilibrated with 10mM-sodium citrate, pH 5.5. The column was washed with 20ml of 10mM-sodium citrate, pH 5.5, and eluted with a discontinuous NaCl gradient of the same buffer containing 0.22M-, 0.38M- or 1.0M-NaCl; 1.0ml fractions were collected. Acrosin eluted from the column was concentrated (Amicon PM 10 membrane), diluted with 2 vol. of 10mM-sodium citrate, pH 5.5, and rechromatographed as described above.

DEAE-cellulose chromatography

Acrosin eluted from CM-cellulose was applied to a DEAE-cellulose column (2.0cm $\times$ 14.0cm) previously equilibrated with 50mM-sodium phosphate, pH 7.2. Acrosin was eluted with the same buffer (flow rate 12ml/h, maintained with a peristaltic pump); 1.0ml fractions were collected. Acrosin eluted from DEAE-cellulose was pooled, adjusted to pH 2.8 (1M-HCl) and made 0.02% (w/v) with respect to Na₂N₃. This preparation was used for analysis of enzyme properties.

Purification of human trypsin

Human trypsin was purified from pancreas (80g) as described by Travis & Roberts (1969), with the following modifications: (1) Sephadex G-50 (fine grade) rather than Sephadex G-75 was used (flow rate 2 ml $\cdot$ cm⁻² $\cdot$ h⁻¹); (2) SP-Sephadex C-50 rather than SE-Sephadex (sulphoethyl-Sephadex) C-25 was used; and (3) buffers contained 5mM- rather than 50mM-CaCl₂.

The method included isolation of trypsinogen from frozen (stored at -20°C) pancreas by acid extraction (0.125M-H₂SO₄) and (NH₄)₂SO₄ fractionation (20–80% saturation). The (NH₄)₂SO₄ was removed by dialysis against 100 vol. of 1mM-HCl, and trypsinogen was activated to trypsin by incubation at 37°C at pH 8.0 (50mM-Tris/HCl) for 2h. Trypsin was purified by SP-Sephadex chromatography at pH 2.6 and at pH 5.1 with discontinuous NaCl gradients (Travis & Roberts, 1969). Trypsin, which was eluted from SP-Sephadex at pH 5.1, was adjusted to pH 3 (1M-HCl) and was used for analyses.

Protein determination

Protein concentration was measured by the method of Lowry *et al.* (1951), by the method of Murphy & Kies (1960), or by reaction with fluorescamine (Udenfriend *et al.*, 1972). Bovine serum albumin was used as a protein standard.

Electrophoresis and molecular-weight determination

Acrosin was subjected to analytical polyacrylamide-gel electrophoresis at pH 4.3 (Brewer & Ashworth, 1969). Approx. 70 μ g of protein was applied to the gels (0.3cm $\times$ 10cm). After electrophoresis (4mA/gel; 5h at 4°C) the gels were stained for protein with Coomassie Brilliant Blue (Weber & Osborn, 1969) or stained for amidase activity with Bz-DL-Arg-NNap and Fast Garnet GBC salt as described by Garner (1975). Protein was detected after destaining with 10% (v/v) acetic acid in 50% (v/v) methanol (15–18h), and was quantified at 650nm with a Gilford model J gel scanner attached to a Gilford model 250 recording spectrophotometer. Amidase activity was detected as a deep-purple stain after removal of excess dye with several changes of 7% (v/v) acetic acid.

Acrosin was subjected to SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). The pH of the acrosin was adjusted to 7.6 (1M-NaOH). SDS and mercaptoethanol were added such that their final concentrations were 1% (w/v). The solution was incubated at 37°C for 2h, and dialysed against 1000 vol. of 10mM-sodium phosphate, pH 7.6, containing 0.1% (w/v) SDS and 0.1% (w/v) mercaptoethanol. The solution was concentrated approx. 15-fold with a Sartorius collodion membrane, mixed with glycerol and Bromophenol Blue (Weber & Osborn, 1969) and applied to the gel (10% acrylamide, 0.25% *NN*-methylenebisacrylamide). After electrophoresis (6h at 23°C; 5mA/gel), the gels were stained with Coomassie Brilliant Blue for 3h. The molecular weight of acrosin was estimated from a standard curve of mobility versus log molecular weight, with the following proteins: bovine serum albumin, *M_r* 68 000 (Tanford *et al.*, 1967); ovalbumin (egg white), *M_r* 45 000 (Castellino & Barker, 1968); bovine pancreatic trypsin, *M_r* 23 300; and bovine haemoglobin, *M_r* 15 500 (Weber & Osborn, 1969; all values are for the monomeric forms of the proteins).

The molecular weights of human acrosin and human trypsin were also estimated by Sephadex chromatography on Sephadex G-150 (flow rate = 5.8 ml $\cdot$ cm⁻² $\cdot$ h⁻¹) and Sephadex G-50 (flow rate = 2.0 ml $\cdot$ cm⁻² $\cdot$ h⁻¹) respectively. The columns were calibrated with proteins of known molecular weight aldolase, *M_r* 158 000 (only for Sephadex G-150) and ribonuclease A, *M_r* 13 700; ovalbumin, *M_r* 45 000; chymotrypsinogen A, *M_r* 25 000]. The molecular weights of human acrosin and trypsin were estimated from a standard curve of log molecular weight versus $(V_e - V_0)/(V_t - V_0)$, where *V_e* = elution volume, *V₀* = void volume; and *V_t* = total column volume.

Determination of apparent *pI*

Polyacrylamide-gel isoelectric focusing was car-

ried out with Bio-Lyte 3/10 and Bio-Lyte 8/10 ampholytes, according to instructions supplied by Bio-Rad Laboratories. After electrophoresis (21h, 20 V/cm of gel, 4°C), the gels were cut into 0.5 cm sections. Each section was placed in 1 ml of 0.1 M-NaCl and homogenized with a glass/Teflon homogenizer and centrifuged at 1000 *g* for 15 min. Enzyme activity and the pH of each supernatant were determined.

Active-site titration

The functional molarities of human acrosin and trypsin were determined by fluorimetric titration with 4-methylumbelliferyl *p*-guanidinobenzoate (Coleman *et al.*, 1976). Trypsin (2–20 milliunits of Bz-Arg-OEt-hydrolysing activity) was added directly to 0.1 μ M-4-methylumbelliferyl *p*-guanidinobenzoate in 50 mM-sodium phosphate, pH 7.5, in a total volume of 3.0 ml. The fluorescence intensity at an excitation wavelength of 366 nm and an emission wavelength of 444 nm (uncorrected) resulting from the addition of enzyme was compared with that obtained for known concentrations of 4-methylumbelliferone. Acrosin (10–20 milliunits of Bz-Arg-OEt-hydrolysing activity) was added to 0.5 μ M-4-methylumbelliferyl *p*-guanidinobenzoate in 0.25 M-sodium phosphate, pH 7.5, in a total volume of 0.6 ml. After incubation for 10 min at ambient temperature, the volume was made to 3.0 ml with water, and the fluorescence intensity was determined. The molarity of active acrosin and trypsin was obtained, assuming a 1:1 stoichiometry between active site and 4-methylumbelliferone released.

Kinetic studies

Reactions were carried out at 25°C in the presence of 50 mM-Tris/HCl, pH 8.0, in a volume of 1.0 ml. Michaelis constants (K_m) and k_{cat} values were determined from plots of reciprocal velocity versus reciprocal substrate concentration (Lineweaver & Burk, 1934). Wavelengths and changes in absorbance corresponding to the hydrolysis of 1.0 μ mol of substrate were as follows: Bz-Arg-OEt, 253 nm, 1.15 $\text{mm}^{-1} \cdot \text{cm}^{-1}$ (Schwert & Takenaka, 1955); Bz-Arg-OMe, 253 nm, 1.15 $\text{mm}^{-1} \cdot \text{cm}^{-1}$; Tos-Arg-OMe, 247 nm, 1.27 $\text{mm}^{-1} \cdot \text{cm}^{-1}$ (Walsh, 1970); and Bz-DL-Arg-Nan, 405 nm, 9.9 $\text{mm}^{-1} \cdot \text{cm}^{-1}$ (Schleuning & Fritz, 1976). Hydrolysis of Bz-DL-Arg-NNap was measured spectrofluorimetrically (Gray *et al.*, 1977). Hydrolysis was quantified with β -naphthylamine standards.

Inhibition of acrosin or trypsin was determined by measuring the difference in activity (Bz-Arg-OEt hydrolysis) after incubation of enzyme (in approximately equimolar concentrations) for 10 min with and without inhibitor.

Mixed-substrate experiments

Acrosin-catalysed reaction velocities were determined in the presence of Bz-Arg-OEt, Bz-Arg-Nan or both. The ratio (R_v) of observed reaction velocity in the presence of both substrates to the sum of velocities observed in the presence of each substrate alone was calculated (Dixon & Webb, 1964). If separate enzymes were responsible for the metabolism of each substrate, R_v would approach 1.0. If the same enzyme were responsible for the metabolism of both substrates, R_v would (theoretically) equal 0.66 when both substrates are used at their K_m concentrations.

Results

Acrosin and proacrosin contents of human spermatozoa

Before proacrosin activation, extracts from fresh spermatozoa contained 0.63 ± 0.15 munits (Bz-Arg-OEt hydrolysis) of activity/ 10^6 spermatozoa ($n = 9$). Enzyme activity increased to 9.8 ± 1.0 munits/ 10^6 spermatozoa ($n = 7$) after incubation at pH 8. Maximal activation occurred within 7 min. Proacrosin comprised about 94% of the total activity. In contrast, frozen spermatozoa contained no proacrosin. Approx. 0.5 munit of acrosin/ 10^6 spermatozoa was extracted from frozen semen (Table 1). No increase in activity was observed after 120 min incubation at pH 8.

Purification of human acrosin

Acrosin was purified over 300-fold by sequential use of Sephadex, CM-cellulose and DEAE-cellulose chromatography, with a yield of 44% of the total activity (Table 1). In all chromatographic procedures, acrosin was eluted as a single peak of activity, as measured by Bz-Arg-OEt hydrolysis. Acrosin was eluted from CM-cellulose with 0.38 M-NaCl. Repetition of the CM-cellulose chromatography yielded an additional 3.8-fold purification. DEAE-cellulose chromatography resulted in a slight increase in total activity, with an additional 1.5-fold purification.

A major protein band was seen after polyacrylamide-gel electrophoresis (pH 4.3), with R_F 0.36 (Fig. 1). This accounted for approx. 90% of the total protein and contained amidase activity, as evidenced by the presence of an activity stain (R_F 0.35 ± 0.02 ; $n = 3$). A minor protein band ($R_F = 0.28$) was also noted (Fig. 1).

A major protein band with an apparent molecular weight of 72 000 was observed after SDS/polyacrylamide-gel electrophoresis. A minor band with an apparent molecular weight of 47 200 was also noted. Approx. 88% of the recovered radioactivity was associated with a M_r -72 000 component after SDS/polyacrylamide-gel electrophoresis

Table 1. *Purification of human acrosin from washed spermatozoa*

Details of the enzyme purification are presented in the text. Values represent the averages $\pm$ s.d. for five separate purifications, and have been normalized to an initial spermatozoa count of $60 \times 10^6/\text{ml}$ (250 ml starting material).

Step	Total protein (mg)	Specific activity (μmol of Bz-Arg-OEt hydrolysed/min per mg of protein)	Purification	Total activity (μmol of Bz-Arg-OEt hydrolysed/min)	Recovery (%)
Washed spermatozoa	323 ± 78	0.02	1	7.3 ± 0.8	100
Acetic acid extract	50 ± 14	0.15	6	7.4 ± 2.5	100
Gel filtration	2.8 ± 0.8	1.50	65	4.2 ± 1.2	57
CM-cellulose chromatography no. 1	0.46 ± 0.06	8.48	369	3.9 ± 0.8	53
CM-cellulose chromatography no. 2	0.085 ± 0.026	31.76	1381	2.7 ± 0.4	37
DEAE-cellulose chromatography	0.065 ± 0.014	49.23	2140	3.2 ± 0.7	44

Fig. 1. *Distribution of Coomassie Brilliant Blue-reactive material in human α -acrosin preparation after polyacrylamide-gel electrophoresis*

Human α -acrosin (obtained after DEAE-cellulose chromatography) was concentrated approx. 15-fold with the aid of a Sartorius collodion bag. Approx. $70 \mu\text{g}$ of protein was applied to polyacrylamide-gel tubes (pH 4.3), and the sample was electrophoresed, as described in the Experimental section. After electrophoresis, the gels were removed from their tubes and stained with Coomassie Brilliant Blue. After destaining the gels, the intensity of stained material was quantified with a Gilford gel scanner (650 nm). Additional details are presented in the Experimental section. The R_F of the major peak at 650 nm is 0.36.

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of human α -acrosin after reaction with $[^3\text{H}]\text{iPr}_2\text{P-F}$*

$[^3\text{H}]\text{iPr}_2\text{P-F}$ was prepared from 250 mM non-radioactive $\text{iPr}_2\text{P-F}$ (in propan-2-ol) and $0.21 \text{ mM-[}^3\text{H(n)}\text{]iPr}_2\text{P-F}$ (sp. radioactivity 0.9 Ci/mmol) such that the final concentration was 10 mM (final sp. radioactivity $45.0 \mu\text{Ci}/\mu\text{mol}$). Human α -acrosin (approx. 0.02 nmol of active enzyme as determined by active-site titration) was incubated with $[^3\text{H}]\text{iPr}_2\text{P-F}$ at pH 8 (50 mM-Tris/HCl) for 20 min at ambient temperature (2.0 ml total volume). After incubation, the inactivated acrosin solution was made 1% (w/v) and 1% (v/v) with respect to SDS and mercaptoethanol respectively. The resulting solution was processed, as described in the Experimental section, for SDS/polyacrylamide-gel electrophoresis. After electrophoresis, gels were cut into 0.5 cm slices. Each gel slice was dissolved in 0.5 ml of $50\% \text{ H}_2\text{O}_2$. Bray's (1960) scintillator was added, and the radioactivity of each slice was determined with a Beckman model LS 230 liquid-scintillation spectrometer. The data were plotted as d.p.m. (assuming a counting efficiency of 39%, as determined by the addition of internal ^3H -labelled toluene) versus distance from the cathode end of the gel.

of acrosin inactivated with $[^3\text{H}]\text{iPr}_2\text{P-F}$ (Fig. 2). Mixed-substrate experiments indicated that only one enzyme was responsible for the observed esterase and amidase activities (Table 2).

Purification of human trypsin

Acid extraction and $(\text{NH}_4)_2\text{SO}_4$ fractionation yielded 12.9 units of trypsin activity per g of frozen human pancreas (Bz-Arg-OEt hydrolysis). Trypsin appeared as single peaks of activity in all chromatographic procedures. The purification scheme (Table 3) resulted in a nearly 200-fold purification over the

activity observed in the activated $(\text{NH}_4)_2\text{SO}_4$ fraction. Trypsin thus purified was stable at pH 3 (4°C) for up to 4 months.

Enzyme properties: comparison of human acrosin with human trypsin

Isoelectric focusing of human acrosin indicated a pI of 9.1 ± 0.2 ($n = 3$). In contrast, most of the

esterolytic activity of the trypsin preparation ($>90\%$) focused at $pI 8.2 \pm 0.1$ ($n = 5$).

The optimal pH for human acrosin and trypsin activities was determined by assaying the enzymes in the presence of 0.5 mM-Bz-Arg-OEt over the pH range 4.0–11.0, in increments of approx. 0.5 unit, at a constant ionic strength of 0.1. Maximal acrosin and trypsin activities were observed at pH 8.0 and pH 7.8 respectively (Table 4).

As estimated by SDS/polyacrylamide-gel electrophoresis, the molecular weight of human acrosin from frozen semen was $7.2 (\pm 0.2) \times 10^4$ ($n = 3$). This estimate agrees with $6.8 (\pm 0.3) \times 10^4$ ($n = 6$) obtained by Sephadex G-150 chromatography. An apparent molecular weight of $2.1 (\pm 0.3) \times 10^4$ ($n = 3$) was determined for human pancreatic trypsin by chromatography on Sephadex G-50 (Table 4).

Bz-Arg-OMe and Bz-Arg-OEt had the lowest Michaelis constants with acrosin, whereas the K_m for Tos-Arg-OMe was approximately one order of magnitude higher (Table 5). In contrast, the Michaelis constants with human trypsin were similar for all three esters. Although k_{cat} values for all esters were similar with acrosin, k_{cat} of Tos-Arg-OMe was higher than that of either Bz-Arg-OMe or Bz-Arg-OEt with human trypsin. Esters were better substrates than amides for acrosin, based on calculated first-order rate constants derived from V_{max}/K_m .

First-order rate constants of the substrates with acrosin and human trypsin indicated similar substrate specificities of the two enzymes, with one notable exception: the highest first-order rate constant with human trypsin, calculated for Tos-Arg-OMe, was nearly two orders of magnitude higher than that obtained with acrosin. In contrast, Bz-Arg-OMe had the highest first-order rate constant with acrosin.

Data for bovine trypsin have been included for comparison (Table 5). Although the substrate specificities for bovine and human trypsin are similar, the first-order rate constant for Bz-DL-Arg-Nan hydrolysis is approximately one order of

Table 2. *Mixed-substrate experiments*

Reaction velocities were determined in the presence of 50 mM-Tris/HCl, pH 8, 0.5 μ g of enzyme protein (acrosin), and Bz-Arg-OEt and/or Bz-DL-Arg-Nan as indicated. The rate of Bz-Arg-OEt hydrolysis with or without Bz-DL-Arg-Nan was determined spectrophotometrically at 253 nm. Hydrolysis of Bz-DL-Arg-Nan with or without Bz-Arg-OEt was determined at 405 nm. Bz-Arg-OEt hydrolysis was not detectable at 405 nm. The ratio R_v represents the ratio of the overall velocity in the presence of both substrates to the sum of the velocities observed for each individual substrate. Theoretical R_v values were calculated by assuming that: (1) in the presence of one enzyme, each substrate acts as a competitive inhibitor of the other, and (2) in the presence of two enzymes, the amidase and esterase activities are additive. See the Experimental section for further details.

[Bz-Arg-OEt] (μ M)	[Bz-DL-Arg-Nan] (μ M)	Activity (μ mol of substrate/ min per mg of protein)
—	250	0.55*
—	250	0.70†
25	—	13.05†
25	250	0.45*
25	250	7.60†

R_v		Observed
Theoretical		
One enzyme	Two enzymes	
0.43†	1.0	0.55†
0.76*	1.0	0.82*

* Reaction measured at 405 nm.

† Reaction measured at 253 nm.

Table 3. *Purification of trypsin from frozen human pancreas*

Details of enzyme purification are presented in the text. Values represent the average of two separate purifications and have been normalized to activity recovered from 80 g of pancreatic tissue.

Step	Total protein (mg)	Specific activity (μ mol of Bz-Arg-OEt hydrolysed/min per mg of protein)	Purification	Total activity (μ mol of Bz-Arg-OEt hydrolysed/min)	Recovery (%)
Activated $(NH_4)_2SO_4$	847	1.22	1	1033	100
Sephadex G-50	42.1	17.1	14	720	70
SP-Sephadex, pH 2.6	4.6	81.8	67	376	36
SP-Sephadex, pH 5.0	1.52	243	199	370	36

Table 4. *Comparison of some physical and catalytic properties of human acrosin and human trypsin*

Details of the determination of the indicated properties of acrosin and trypsin are described in the Experimental section. Values represent the average $\pm$ S.D. for the numbers of separate determinations indicated in parentheses.

	Acrosin	Trypsin
Molecular weight	68000 $\pm$ 3000 ($n = 6$)* 72000 $\pm$ 4000 ($n = 3$)†	21000*
pI	9.1 $\pm$ 0.2 ($n = 3$)	8.2 $\pm$ 0.1 ($n = 5$)‡
pH optimum	8.0	7.8
Catalytic-centre activity for Bz-Arg-OEt (μ mol of Bz-Arg-OEt hydrolysed/s per μ mol of enzyme)	110 $\pm$ 10 ($n = 3$)	73 $\pm$ 10 ($n = 3$)

* Molecular weight was determined by Sephadex chromatography (for details, see the Experimental section).

† Molecular weight was determined by SDS/polyacrylamide-gel electrophoresis (for details, see the Experimental section).

‡ Represents greater than 90% of recovered activity.

Table 5. *Michaelis constants of synthetic substrates: comparison between human acrosin, human trypsin and bovine trypsin*

Substrate	Human acrosin		Human trypsin		Bovine trypsin	
	K_m (μ M)*	k_{cat} (s^{-1})*	K_m (μ M)*	k_{cat} (s^{-1})*	K_m (μ M)†	k_{cat} (s^{-1})†
Bz-Arg-OEt	36 $\pm$ 9	87 $\pm$ 3	12 $\pm$ 3	58 $\pm$ 4	4.3	14.6
Bz-Arg-OMe	20 $\pm$ 1	80 $\pm$ 2	5 $\pm$ 2	42 $\pm$ 5	—	—
Tos-Arg-OMe	320 $\pm$ 10	68 $\pm$ 3	10 $\pm$ 3	194 $\pm$ 10	12.5	100
Bz-DL-Arg-Nan	180 $\pm$ 20	3.7 $\pm$ 0.7	—	—	—	—
Bz-DL-Arg-NNap	280 $\pm$ 50	2.8 $\pm$ 0.3	426 $\pm$ 22	3.2 $\pm$ 0.4	939	0.61
Ac-Tyr-OEt	—	0‡	—	0‡	42000	14.5

* Values are expressed as average $\pm$ S.D. for four separate determinations.

† Data taken from Walsh (1970).

‡ No detectable activity observed at substrate concentrations of 5 mM.

Table 6. *Effect of various inhibitors on human acrosin and human trypsin*

Either trypsin or acrosin (approx. 2 pmol, as determined by active-site titration) was preincubated with each inhibitor at the indicated concentration at pH 8.0 (50 mM-Tris/HCl) for 10 min at ambient temperature before initiation of reactions with 0.5 mM-Bz-Arg-OEt. Results are expressed as percentage inhibition of rates obtained after preincubation of enzymes in the absence of inhibitor.

Inhibitor	Inhibition (%)	
	Acrosin	Trypsin
Soya-bean trypsin inhibitor (4 nM)	18.0	52.9
Tos-Lys-CH ₂ Cl (0.5 mM)	17.0	82.0
iPr ₂ P-F (0.1 mM)	20.3	52.8
D-Fructose (0.1 M)	47.0	7.0
D-Arabinose (0.1 M)	36.0	0.0

magnitude higher with human than with bovine trypsin.

Soya-bean trypsin inhibitor and iPr₂P-F inhibited human acrosin and human trypsin (Table 6). However, these inhibitors were less reactive toward acrosin. On the other hand, D-fructose and D-arabinose inhibited acrosin, but had no effect on human trypsin (Table 6).

Discussion

The present study has compared a single molecular form of human acrosin (α -acrosin) with human trypsin, with respect to physical and kinetic parameters. In freshly ejaculated human spermatozoa, approx. 90% of the total acrosin was found in the zymogen form (proacrosin). It appears that freezing caused the destruction of proacrosin. These data are in agreement with those of Goodpasture *et al.* (1981).

The present purification scheme was different from that used by Gilboa *et al.* (1973), in that anion-exchange chromatography was used. The final specific activity was approx. 12% higher than that previously published (Table 1). However, the acrosin isolated by Gilboa *et al.* (1973) may have been contaminated by multiple molecular-weight forms, since the semen was not subjected to prior freezing. This possibility is strengthened by their report of a shoulder on the peak of activity after Sephadex chromatography. The destruction of proacrosin after freezing precludes the formation of multiple molecular forms of acrosin, as previously described (Schleuning *et al.*, 1976; Tobias & Schumacher, 1977). A single symmetrical peak of activity was obtained from each chromatographic step. Acrosin in frozen spermatozoa is a stable high-molecular-weight form of the enzyme (M_r 70 000; Table 4).

Polyacrylamide-gel electrophoresis indicated approx. 90% purity with respect to protein content (Fig. 1). On the basis of active-site titration, approx. 75% of the protein was active α -acrosin. The remainder of the protein most probably represents inactive acrosin resulting from autolysis. Further purification and/or elaboration of the homogeneity of acrosin preparations was not practical, owing to extremely low protein yields (i.e. 65 μ g) from large quantities (250 ml) of human semen, representing pooled ejaculates from over 75 donors.

α -Acrosin contained no contaminating esterase or amidase activities. Mixed-substrate experiments (Table 2) were consistent with the presence of only one enzyme, which hydrolysed amides and esters. Moreover, analysis of kinetic data by double-reciprocal plots showed no deviation from linearity. These findings indicate the presence of only one enzyme with esterolytic and amidolytic activities.

Several modifications were made to the method of Travis & Roberts (1969) for the purification of human pancreatic trypsin, resulting in a 200-fold purification of activity. This is compared with a 14-fold purification reported by Travis & Roberts (1969), or a 16-fold purification reported by Feinstein *et al.* (1974).

Two forms of trypsin are present in human pancreas. An anionic trypsin (Mallory & Travis, 1973) represents a minor component and differs from the cationic trypsin purified by Travis & Roberts (1969), in that it contains more aspartic acid and glutamic acid residues, is labile at pH 3 and is more sensitive to inhibition by soya-bean trypsin inhibitor. Feinstein *et al.* (1974) also isolated two trypsins from human pancreas, designated trypsins I and II. On the basis of amino acid composition, molecular weight and enzyme stability, trypsins I and II correspond to cationic and anionic trypsins respectively. Trypsin isolated in the present work is probably trypsin I, or cationic trypsin, since it is

stable at acid pH and has a molecular weight in good agreement with that reported for trypsin I (Feinstein *et al.*, 1974) and cationic trypsin (Travis & Roberts, 1969).

Although human α -acrosin can be broadly classified as a trypsin-like enzyme, major physical and kinetic differences exist. A notable difference between acrosin and trypsin was found in their apparent molecular weights. Human α -acrosin has a molecular weight of approx. 70 000 (Table 4). This is in good agreement with that of 75 000 reported by Gilboa *et al.* (1973). The molecular weight of α -acrosin is in contrast with the molecular weight of 21 000 for trypsin (Table 4). The molecular weight for human trypsin is similar to that reported for other species (Walsh, 1970).

The isoelectric point of human trypsin was lower than that of bovine trypsin or human acrosin (Table 4). Owing to the similarity in amino acid composition between human and bovine trypsins (Mallory & Travis, 1973), these data suggest substantial differences in the tertiary structure of the two enzymes. From the reported amino acid composition of bovine (Walsh & Neurath, 1964) and human I (Feinstein *et al.*, 1974) trypsins, the isoelectric points of these enzymes are calculated to be 6.9 and 6.8 respectively (Tanford & Hauenstein, 1956). The relatively low pI obtained for human trypsin suggests that human trypsin has fewer sequestered acidic amino acid residues than bovine trypsin.

Bz-Arg-OEt is the only substrate for which a K_m has been previously reported with human acrosin. A value of 3.6×10^{-3} M reported herein is in excellent agreement with the value obtained with crude human acrosin preparations (Bhattacharyya *et al.*, 1976). Of the esters examined, Tos-Arg-OMe had the highest K_m (Table 5). This may be due to electron-withdrawing effects of the tosyl moiety, rendering the ester more resistant to nucleophilic attack. Similar K_m values for all esters were obtained with human trypsin, providing further evidence for kinetic differences between the two enzymes.

With human trypsin, k_{cat} values for the Bz-Arg-OMe and Bz-Arg-OEt were similar, whereas k_{cat} values for Tos-Arg-OMe and Bz-Arg-OMe were dissimilar. These data suggest that deacylation is the rate-limiting step of ester hydrolysis by human trypsin, as proposed for bovine trypsin (Roberts & Elmore, 1974; Kraut, 1977). In contrast, k_{cat} values for all three esters were similar with acrosin (Table 5), suggesting that deacylation may not be rate-limiting for this enzyme. Further experimentation would be required to clarify this point. When human acrosin is compared with bovine trypsin, the specificity constants (k_{cat}/K_m ; Roberts & Elmore, 1974) indicate that Bz-DL-Arg-Nan is a better substrate for acrosin (Table 5). On the other hand, the substrate is equally hydrolysed by human

acrosin and human trypsin. These data illustrate the importance of comparing enzymes from the same species.

Tos-Lys-CH₂Cl, iPr₂P-F and soya-bean trypsin inhibitor inhibited human acrosin and trypsin (Table 6). Acrosin was consistently less sensitive than trypsin to these inhibitors. Soya-bean trypsin inhibitor did not totally inhibit human trypsin at inhibitor/enzyme ratios greater than 1:1; this further indicates that human cationic trypsin (Mallory & Travis, 1973) is the enzyme described in the present study.

Human acrosin is sensitive to inhibition by monosaccharides, including D-fructose and D-arabinose (Anderson *et al.*, 1980). Although the mechanism of inhibition is not known, the glycoprotein nature of acrosin (Schleuning & Fritz, 1974, 1976; Brown & Hartree, 1978) may be partly responsible for this effect. The lack of effect of D-fructose or D-arabinose on trypsin further indicates that acrosin and trypsin differ in inhibitor sensitivity.

The present data provide convincing evidence that human acrosin differs from human trypsin. Although acrosin resembles trypsin in many respects, the two enzymes are sufficiently different in physical and catalytic properties that studies on acrosin are warranted. Such studies may provide a better understanding of the function of α -acrosin in enzymic processes related to human reproduction.

References

- Anderson, R. A., Oswald, C., Leto, S. & Zaneveld, L. J. D. (1980) *Biol. Reprod.* **22**, 1079–1082
- Bhattacharyya, A. K., Zaneveld, L. J. D., Dragoje, B., Schumacher, G. F. B. & Travis, J. (1976) *J. Reprod. Fertil.* **47**, 97–100
- Bhattacharyya, A. K., Goodpasture, J. C. & Zaneveld, L. J. D. (1979) *Am. J. Physiol.* **237**, E40–E44
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Brewer, J. M. & Ashworth, R. B. (1969) *J. Chem. Educ.* **46**, 41–45
- Brown, C. R. & Hartree, E. F. (1978) *Biochem. J.* **175**, 227–238
- Castellino, F. J. & Barker, R. (1968) *Biochemistry* **7**, 2207–2217
- Coleman, P. L., Latham, H. G. & Shaw, E. N. (1976) *Methods Enzymol.* **45**, 12–26
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, pp. 242–247. Longmans Green, London
- Feinstein, G., Hofstein, R., Koifmann, J. & Sokolovsky, M. (1974) *Eur. J. Biochem.* **43**, 569–581
- Fink, E. & Wendt, V. (1978) *J. Reprod. Fertil.* **53**, 75–76
- Fink, E., Schiessler, H., Arnhold, M. & Fritz, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1633–1637
- Fritz, H., Heimbürger, N., Meier, M., Arnhold, M., Zaneveld, L. J. D. & Schumacher, G. F. B. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1953–1956
- Gaddum, P. & Blandau, R. J. (1970) *Science* **170**, 749–751
- Garner, D. L. (1975) *Anal. Biochem.* **67**, 688–694
- Garner, D. L. & Cullison, R. F. (1974) *J. Chromatogr.* **92**, 445–449
- Gilboa, E., Elkana, Y. & Rigbi, M. (1973) *Eur. J. Biochem.* **39**, 85–92
- Goodpasture, J. C., Zavos, P. M., Cohen, M. R. & Zaneveld, L. J. D. (1981) *J. Reprod. Fertil.* in the press.
- Gray, G. M., Tabiowo, A. & Trotter, M. D. (1977) *Biochem. J.* **161**, 667–675
- Kraut, J. (1977) *Annu. Rev. Biochem.* **46**, 331–358
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 659–666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mallory, P. A. & Travis, J. (1973) *Biochemistry* **12**, 2847–2851
- Meizel, S. & Huang-Yang, Y. H. J. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1145–1150
- Meizel, S. & Mukerji, S. K. (1975) *Biol. Reprod.* **13**, 83–93
- Morton, D. B. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 445–500, North-Holland Publishing Co., Amsterdam
- Multimaki, S. & Niemi, M. (1972) *Int. J. Fertil.* **17**, 43–52
- Murphy, J. B. & Kies, M. W. (1960) *Biochim. Biophys. Acta* **45**, 382–384
- Polakoski, K. L. & Zaneveld, L. J. D. (1976) *Methods Enzymol.* **45**, 325–329
- Polakoski, K. L., Zaneveld, L. J. D. & Williams, W. L. (1972) *Biol. Reprod.* **6**, 23–29
- Roberts, D. V. & Elmore, D. T. (1974) *Biochem. J.* **141**, 545–554
- Schleuning, W.-D. & Fritz, H. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 125–130
- Schleuning, W.-D. & Fritz, H. (1976) *Methods Enzymol.* **45**, 330–342
- Schleuning, W.-D., Hell, R. & Fritz, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 207–212
- Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta* **16**, 570–575
- Stambaugh, R. & Buckley, J. (1968) *Science* **161**, 585–586
- Stambaugh, R. & Buckley, J. (1970) *Biol. Reprod.* **3**, 275–282
- Stambaugh, R. & Buckley, J. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 1184
- Stambaugh, R. & Smith, M. (1974) *Science* **186**, 745–746
- Tanford, C. & Hauenstein, J. D. (1956) *J. Am. Chem. Soc.* **78**, 5287–5291
- Tanford, C., Kawahara, K. & Lapanje, S. (1967) *J. Am. Chem. Soc.* **89**, 729–736
- Tobias, P. S. & Schumacher, G. F. B. (1977) *Biochem. Biophys. Res. Commun.* **74**, 434–439
- Travis, J. & Roberts, R. C. (1969) *Biochemistry* **8**, 2884–2889
- Udenfriend, S., Stein, S., Bohlen, P., Dauman, W., Leimgruber, W. & Weigle, M. (1972) *Science* **178**, 871–872

- Walsh, K. A. (1970) *Methods Enzymol.* **19**, 41–63
- Walsh, K. & Neurath, H. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **52**, 884–889
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Zahler, W. L. & Polakoski, K. L. (1977) *Biochim. Biophys. Acta* **480**, 461–468
- Zaneveld, L. J. D., Robertson, R. T. & Williams, W. L. (1970) *FEBS Lett.* **11**, 345–347
- Zaneveld, L. J. D., Polakoski, K. L. & Williams, W. L. (1972) *Biol. Reprod.* **6**, 30–39
- Zaneveld, L. J. D., Polakoski, K. L. & Williams, W. L. (1973) *Biol. Reprod.* **9**, 219–225