Caprine acrosin

Purification, characterization and proteolysis of the porcine zona pellucida

Daniel M. HARDY,* Andre F. M. SCHOOTS† and Jerry L. HEDRICK

Department of Biochemistry and Biophysics, University of California, Davis, CA 95616, U.S.A.

Acrosin purified from an acidic extract of ejaculated goat spermatozoa migrated as a single 42000- M_r band in SDS/polyacrylamide-gel electrophoresis. Reduction and alkylation of caprine acrosin produced two polypeptides, one of M_r 40000 (heavy chain) and the other of M_r 3700 (light chain). The light chain purified by reversed-phase h.p.l.c. was a glycosylated octadecapeptide with an amino acid sequence similar to that of the *N*-terminal 18 residues of porcine acrosin light chain (78 % positional identity). The sequence of the *N*-terminal 37 amino acids of purified caprine acrosin heavy chain is similar to that of porcine acrosin heavy chain (70 % positional identity through 37 residues). Studies with synthetic substrates and synthetic and natural proteinase inhibitors confirmed both the specificity of the purified proteinase for Arg-Xaa and Lys-Xaa bonds and a serine-proteinase mechanism. Purified caprine acrosin hydrolysed the 90 kDa and 65 kDa components, but did not hydrolyse the 55 kDa component of the porcine zona pellucida. The action of the enzyme on the porcine zona pellucida was indistinguishable from that previously reported for porcine acrosin.

INTRODUCTION

Acrosin (EC 3.4.21.10) is a serine proteinase with specificity for Lys-Xaa and Arg-Xaa bonds (reviewed in [1]). Acrosins have been purified to homogeneity from acidic extracts of washed porcine [2–4], ovine [5], and murine [6] spermatozoa. Both acrosin and its zymogen, proacrosin, are glycoproteins [7–9]. Structural studies of porcine acrosin showed that the enzyme has a two-chain structure; the 4200- M_r porcine acrosin light chain is 23 amino acids in length, contains *N*-linked carbohydrate, and is attached via two disulphide bonds to a 37000- M_r heavy chain [9]. The *N*-terminal amino acid sequence of the porcine acrosin heavy chain is similar to those of other serine proteinases, thus suggesting homology with these enzymes [10]. Likewise, the sequence of the porcine acrosin light chain is similar to the sequences of activation peptides of several serine proteinases, suggesting that limited proteolysis of a single polypeptide to produce the light and heavy chains is the mechanism of proacrosin activation [9].

Studies using proteinase inhibitors implicate acrosin in multiple fertilization events, including membrane fusion [11,12] and acrosomal matrix dispersal [13–15] in the acrosome reaction, and sperm binding to [16] and penetration of [17,18] the egg zona pellucida (ZP). A role for acrosin in ZP penetration is further supported by observations that one (90 kDa and its derived product 65 kDa) of the three glycoprotein families comprising the porcine ZP are porcine acrosin substrates [19–22].

Animal fertilization is largely species-specific [23–25].

This specificity is due in part to species specificity of sperm interaction with the egg ZP [23]. Since acrosin functions in sperm-ZP interactions, inter-species comparisons of components of the acrosin system may provide important insights into the species specificity of this interaction. In the present paper we report the purification and characterization of caprine acrosin, and compare physicochemical and enzymic properties of caprine acrosin, including *N*-terminal amino acid sequence and action on the porcine ZP, to those published for porcine acrosin. A preliminary account of some aspects of this work was previously reported [26].

EXPERIMENTAL

Materials

Unless otherwise specified, chemicals were of reagent grade. The porcine ZP was prepared from freshly frozen ovaries as described [27]. Goat (*Capra hircus*) semen was collected, using an artificial vagina, from fertile bucks during the normal breeding season (September–December). Porcine acrosin, purified as described by Muller-Esterl *et al.* [4], was kindly given by Dr. U. A. Urch, University of California, Davis, CA, U.S.A.

Enzyme assays

Acrosin-mediated amidolysis of Bz-Arg-NNab (1.1 mM in 90 mM-Tris/HCl/45 mM-CaCl₂/0.01 % Triton X-100, pH 8.0) was measured fluorimetrically [28]. When desired, amidolysis of Bz-Arg-NPhNO₂ [3], and

Abbreviations used: α -Tos-F, α -tosyl fluoride; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane; Tos-Phe-CH₂Cl, *N*-tosyl-L-phenylalanylchloromethane; Gu-Bz-PhNO₂, *p*-nitrophenyl *p*-guanidinobenzoate; Tos-Arg-OMe, *N*^ε-tosyl-L-arginine methyl ester; Bz-Arg-OEt, *N*^ε-benzoyl-L-arginine ethyl ester; Bz-Arg-NNab, *N*^ε-benzoyl-L-arginine β -naphthylamide; Bz-Arg-NPhNO₂, *N*^ε-benzoyl-L-arginine *p*-nitroanilide; STI, soybean trypsin inhibitor; OTI, ovomucoid trypsin inhibitor; PAGE, polyacrylamide-gel electrophoresis; ZP, zona pellucida.

* To whom correspondence and reprint requests should be sent.

† Present address: Institute for Land and Water Management Research, P.O. Box 35, 6700 AA Wageningen, The Netherlands.

esterolysis of Bz-Arg-OEt [29] and Tos-Arg-OMe [3] were determined spectrophotometrically. K_m and k_{cat} values were calculated from Lineweaver-Burk plots of the kinetic data, and K_i values were determined by using Dixon [30] plots.

Protein concentrations

Protein concentrations were measured either as described by Lowry *et al.* [31], or with bicinchoninic acid by the method of Smith *et al.* [32], bovine serum albumin being used as the standard in both cases.

Purification of caprine acrosin

Caprine acrosin was purified from goat semen using a method previously reported for purification of porcine sperm acrosin [4], except that Extracti-gel D (Pierce Chemical Co., Rockford, IL, U.S.A.) was used to remove Triton X-100 from the acrosin pool from affinity chromatography, and the enzyme was then further purified by gel filtration using Sephadex G-75. The purified enzyme was freeze-dried and stored at -20°C .

Electrophoresis

SDS/PAGE was performed as described by Laemmli [33]. Two-dimensional PAGE, consisting of isoelectric focusing in the first dimension and SDS/PAGE in the second dimension, was by the method of O'Farrell [34]. Samples for SDS/PAGE and two-dimensional PAGE were either applied to the gels without prior reduction or reduced when desired by adding mercaptoethanol to a final concentration of 5% (v/v) and heating to 100°C for 2 min.

H.p.l.c.

To remove a peptide contaminant which confounded efforts to sequence caprine acrosin, the enzyme (purified as described above) was dissolved at 2 mg/ml in 35% acetonitrile [solvent concentrations are (v/v)] containing 0.1% trifluoroacetic acid, filtered through a $0.45\text{ }\mu\text{m}$ -pore-size nitrocellulose membrane, and separated by h.p.l.c. at 25°C on a C_4 reversed-phase column (Vydac 214TP104; Alltech, Deerfield, IL, U.S.A.). Column elution employed a gradient constructed with 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Gradient compositions are expressed as the percentage of solvent B in solvent A. The column was equilibrated in 35% B and eluted at 1.0 ml/min with a linear 35–40% B gradient over 30 min, followed by a linear 40–100% B gradient over 10 min.

To separate polypeptide chains comprising caprine acrosin, the enzyme was reduced and alkylated (method described below), and then separated by reversed-phase h.p.l.c. as described above, except that the column was equilibrated in 10% B and eluted with a 10–35% B linear gradient over 12.5 min, followed by a 35–40% B linear gradient over the next 30 min.

The relative molecular mass (M_r) of caprine acrosin light chain was determined by gel-filtration h.p.l.c. in 6.0 M-guanidinium chloride [35]. Reduced and alkylated caprine acrosin was applied to a $7.5\text{ mm} \times 30\text{ cm}$ Spherogel-TSK2000-SW ($10\text{ }\mu\text{m}$ particle size) column (Altex, Berkeley, CA, U.S.A.) equilibrated and run at 0.5 ml/min in 0.10 M-sodium phosphate, pH 6.0, containing 6.0 M-guanidinium chloride (Sequal grade; Pierce Chemical Co.) at 25°C . The column was calibrated

by determining the elution times of reduced and alkylated protein standards: soybean trypsin inhibitor (STI; M_r 20100), myoglobin (27200), ribonuclease (13700), aprolinin (6500) and insulin B-chain (3400). A calibration curve was constructed by plotting $\log M_r$ against elution time, and the data were fitted to a line by the least-squares method (correlation of fit: $r^2 = 0.999$).

Reduction and alkylation of caprine acrosin

Caprine acrosin, purified by h.p.l.c. as described above, was dissolved at 10 mg/ml in 0.2 M-Tris/HCl, pH 8.6, containing 1 mM-EDTA and 6 M-guanidinium chloride. The desired quantity of dissolved enzyme (10–20 nmol, 400–800 μg) was reduced under an atmosphere of Ar by adding sufficient dithiothreitol solution (60 mg/ml in Tris/EDTA/guanidinium chloride buffer) to produce a ratio of 240 μmol of dithiothreitol/ μmol of acrosin and incubating for 1 h at 25°C . The pH of the mixture was then adjusted to 7.4 by adding 150 mM- NaH_2PO_4 containing 6 M-guanidinium chloride, and iodoacetamide solution (100 mg/ml in water) was then added to produce a ratio of 520 μmol of iodoacetamide/ μmol of acrosin. After incubating for 30 min at 25°C in the dark, the preparation was separated immediately by h.p.l.c. (as described above) or stored frozen at -20°C .

Amino acid composition

The 3700- M_r caprine acrosin chain (240 pmol) was hydrolysed for 24 h at 100°C in 6 M-HCl, and the hydrolysate was analysed with an automated amino acid analyser.

Amino acid sequencing

Caprine acrosin chains (1–4 nmol), purified by reversed-phase h.p.l.c., were sequenced by automated Edman degradation [36]. The 3700- M_r chain was sequenced by using an Applied Biosystems 470A vapour-phase sequencer, and the 38000- M_r chain was sequenced by using a Beckman 890M liquid-phase sequencer. Liberated amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. on cyano [37] and C_{18} [38] columns.

Hydrolysis of porcine ZP by caprine acrosin

To 300 μg of heat-solubilized [19] porcine ZP in 150 μl of 0.1 M-Tris/HCl, pH 8.0, was added 20 μg of purified caprine acrosin. The mixture was incubated at 25°C , and aliquots (25 μl) were withdrawn at the desired times and denatured by heating in SDS/PAGE sample buffer containing 5% mercaptoethanol. The samples were then analysed by SDS/PAGE.

In a second experiment, 400 μg of heat-solubilized porcine ZP was mixed with caprine acrosin as described above, and the mixture was divided into two halves. One half was incubated for 0 and one for 24 h at 25°C , and then analysed by two-dimensional PAGE.

RESULTS

Purification of caprine acrosin is summarized in Table 1. The purified enzyme migrated primarily as a 42000- M_r band in SDS/PAGE without prior reduction, and as a 40000- M_r band under reducing conditions (Fig. 1). At least one minor contaminant with an M_r lower than 17000 was apparent on heavily overloaded gels.

Table 1. Summary of caprine acrosin purificationAbbreviations: CM, carboxymethyl; pAB, *p*-aminobenzamidine.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%) [‡]	Purification (fold) [§]
Extraction*	320	14	0.19 [†]	23	1.0
Sephadex G-100	130	41	0.48 [†]	67	2.5
Phenyl-Sepharose	61	62	1.0	100	5.3
CM-Sephadex	42	60	1.4	96	7.5
pAB-Sepharose	23	54	2.4	87	12
Extracti-gel D	20	46	2.3	75	12
Sephadex G-75	12	42	3.5	68	18

* From 47 ml of semen containing 1.4×10^{11} spermatozoa.[†] Calculated assuming a total activity of 62 units as determined after phenyl-Sepharose chromatography.[‡] Calculated relative to the yield after phenyl-Sepharose chromatography; the increases in total activity observed after the first two purification steps are likely due to removal of endogenous inhibitors (A. Schoots, unpublished work).[§] Increment in specific activity over that determined after extraction.

N-Terminal amino acid sequencing of caprine acrosin purified through gel filtration on Sephadex G-75 indicated the presence of a non-acrosin contaminant, since a sequence unrelated to those of porcine acrosin [9,10] was obtained in addition to sequences attributable to acrosin. Further purification of caprine acrosin by reversed-phase h.p.l.c. produced four peaks which were eluted at 17, 22, 28 and 43 min (Fig. 2a). The peaks that were eluted at 28 and 43 min were identified as residual

Triton X-100 components by comparison with the behaviour of Triton X-100 alone under the same conditions. Only the peak that was eluted at 22 min possessed acrosin activity (Bz-Arg-NNab hydrolysis). Acrosin purified by h.p.l.c. migrated as a single 42000- M_r band (non-reducing conditions) in SDS/PAGE, and as a single 40000- M_r band under reducing conditions (Fig. 1). Thus the enzyme was homogeneous by electrophoretic criteria.

Reversed-phase h.p.l.c. of reduced and alkylated caprine acrosin resolved two protein peaks eluted with retention times of 10 and 34 min (Fig. 2b). No peak was eluted at 10 min if dithiothreitol was omitted from the reduction and alkylation procedure. The material that was eluted at 34 min migrated as a single band with a lower mobility than that of intact acrosin in SDS/PAGE under non-reducing conditions and co-migrated with acrosin at 40000 M_r under reducing conditions (Fig. 1).

Gel-filtration h.p.l.c. in 6 M-guanidinium chloride of reduced and alkylated caprine acrosin also resolved two protein peaks. The major peak was eluted with an M_r of 40000, and the minor peak was eluted with an M_r of 3700 ± 100 (mean of four determinations $\pm$ s.d.). The 40000- M_r peak material from gel filtration was eluted as a single peak at 34 min in reversed-phase h.p.l.c. (conditions as shown in Fig. 2b), and the 3700- M_r peak material from gel filtration was eluted as a single peak at 10 min in reversed-phase h.p.l.c. under these conditions; thus the polypeptides were homogeneous.

The amino acid sequence of the 3700- M_r polypeptide purified by reversed-phase h.p.l.c. is shown in Fig. 3. The peptide is 18 amino acids long; recovery decreased from 1.6 nmol in cycle 17 to 200 pmol in cycle 18 to less than 100 pmol in cycle 19. In three sequencing attempts using different preparations of the peptide, no amino acid residue was detected in the third cycle. On the final attempt, 4 nmol of the peptide were sequenced; no residue was detectable at greater than 400 pmol in the third cycle, even though yields were 3.6 nmol in the preceding cycle and greater than 2 nmol in subsequent cycles. The amino acid composition of the peptide indicated the presence of four aspartic acid/asparagine residues per 18 total residues. Since only three aspartic acid/asparagine

Fig. 1. SDS/PAGE (12.5% gels) of caprine acrosin

Lane 1, acrosin before reversed-phase h.p.l.c.; lane 2, acrosin further purified by reversed-phase h.p.l.c. (22 min peak; Fig. 2a); lane 3, acrosin chain isolated by reversed-phase h.p.l.c. (34 min peak; Fig. 2b) of reduced and alkylated acrosin. NR, samples (4 μ g each) not reduced; R, reduced samples (2 μ g each). Migration (as M_r) of non-reduced standards is shown on the left, and migration of reduced standards is shown on the right.

Fig. 2. Reversed-phase h.p.l.c. of caprine acrosin

The progress of the elution gradient expressed as percent of acetonitrile in the eluting solvent is indicated by the broken lines. (a) Acrosin (200 µg) purified through Sephadex G-75 chromatography; acrosin activity was eluted at 22 min; (b) 200 µg of reduced and alkylated acrosin.

residues per molecule of peptide were detected by sequencing, the 'missing' residue was tentatively assigned as an asparagine in position 3 (see the Discussion section). Results of amino acid composition analysis of the peptide also indicated the presence of glucosamine. When aligned with positions 1–18 of the sequence of porcine acrosin light chain (Fig. 3), the two sequences are identical in 14 of the 18 positions compared (78% positional identity).

The *N*-terminal amino acid sequence of the 40000-*M_r* polypeptide purified by reversed-phase is shown in Fig. 3. During sequencing, recoveries ranged from 3.9 nmol in cycle 2 to 100–200 pmol in cycles 31–37. When aligned with the *N*-terminal sequence of porcine acrosin heavy chain, the two sequences are identical in 26 out of the 37 positions compared (70% positional identity). In addition, 16 of the 22 positions typically conserved (73%) and all of four invariable positions in this region of serine proteinases are conserved in caprine acrosin.

The effects of four synthetic and two natural proteinase inhibitors on amidase activity (Bz-Arg-NNab hydrolysis) of purified caprine and porcine acrosins are shown in Table 2. Caprine acrosin was inhibited by trypsin inhibitors, but not by the irreversible inhibitor of chymotrypsin, Tos-Phe-CH₂Cl. In comparison, porcine acrosin purified by the same method was generally more sensitive than caprine acrosin to inhibition by trypsin inhibitors (Table 2). Similarly, the *K_i* of benzamidine and of *p*-aminobenzamidine were 10.7 µM and 2.7 µM respectively for caprine acrosin and 4.0 µM and 1.2 µM respectively for porcine acrosin. *K_m* and *k_{cat}* values for four synthetic substrates are presented in Table 3. Collectively, the substrate and inhibitor studies confirm that the enzyme is a serine amidase and esterase with specificity for Lys-Xaa and Arg-Xaa bonds.

Light chains

	1	5	10	15	20																		
Caprine acrosin	R	D	N	T	T	C	D	G	P	C	G	I	R	F	R	Q	N	R					
Porcine acrosin ^a	R	D	N	A	T	C	D	G	P	C	G	L	R	F	R	Q	K	L	E	S	G	M	R

Heavy chains

	1	5	10	15	20	25	30	35																												
CA	I	I	G	G	Q	D	A	A	H	G	S	W	P	W	M	V	S	L	Q	I	F	T	Y	H	N	N	R	R	Y	H	V	C	G	G	S	L
PA ^b	V	V	G	G	M	S	A	E	P	G	A	W	P	W	M	V	S	L	Q	I	F	M	Y	H	N	N	R	R	Y	H	T	C	G	G	I	L
TC ^c	I	V	G	G		A				G	S		P	W	Q	V	S	L				S	G						H	F	C	G	G		L	
I ^c													P																							

Fig. 3. Comparison of caprine acrosin (CA) light and heavy chain amino acid sequences with those of porcine acrosin (PA)

Identical positions are boxed. Abbreviations: TC, typically conserved; I, invariable. Arginine residues in position 18 of the light chain and positions 27 and 28 of the heavy chain, and histidine residues in positions 24 and 30 of the heavy chain, were identified by only one of the two h.p.l.c. methods used, and are therefore unconfirmed. Residue 3 of the caprine acrosin light chain was assigned as asparagine as described in the text. References: ^afrom [9]; ^bfrom [10]; ^cfrom [51].

Table 2. Inhibition of acrosins by synthetic and natural inhibitors

Inhibitor	Concn. (μM)	Incubation time (min)	Inhibition (%)	
			Acrosin ...	Caprine Porcine
$\alpha\text{Tos-F}$	100	60	5	10
	100	180	22	76
	300	240	67	98
Tos-Lys-CH ₂ Cl	100	180	66	100
	300	180	88	100
Tos-Phe-CH ₂ Cl	300	180	0	0
Gu-Bz-PhNO ₂	2	30	100	100
OTI	—*	30	92	100
STI	—*	30	100	100

* Tested at 100-fold excess by weight of inhibitor over enzyme.

Table 3. Kinetic coefficients of caprine-acrosin hydrolysis of synthetic substrates

Abbreviation: ND, not determined.

Substrate	K_m (μM)	k_{cat} (s^{-1})
Esters		
Tos-Arg-OMe	77	31
Bz-Arg-OEt	44	77
Amides		
Bz-Arg-NNab	270	ND
Bz-Arg-NPhNO ₂	110	33

To determine the species specificity of the action of acrosin on the ZP, heat-solubilized porcine ZP was incubated with purified caprine acrosin, and the resultant hydrolysates were analysed by SDS/PAGE. Treatment with caprine acrosin for 1 to 24 h produced essentially the same pattern of hydrolysis products, irrespective of the lengths of the incubations (Fig. 4). ZP components migrating in the 60 000–90 000- M_r range were extensively hydrolysed by 1 h. Components in the 50 000–55 000- M_r range were relatively resistant to proteolysis, even after treatment for 24 h. The specific components hydrolysed were identified by two-dimensional PAGE. Caprine acrosin preferentially hydrolysed the 90 kDa glycoprotein family of the porcine ZP and its derived 65 kDa glycoproteins, leaving the 55 kDa family largely unaffected (Fig. 5).

DISCUSSION

Caprine acrosin was purified from acidic extracts of ejaculated goat spermatozoa. Final purification was by reversed-phase h.p.l.c. The purified caprine acrosin was electrophoretically homogeneous, and migrated with an M_r of 42 000 on SDS/PAGE. Substrate and inhibitor studies confirmed a serine-proteinase mechanism and specificity for Lys-Xaa and Arg-Xaa bonds, as demonstrated previously for porcine acrosin [1].

Reduction and alkylation of caprine acrosin produced two non-identical polypeptides; the two peptides were not separable without prior reduction. Thus caprine

Fig. 4. SDS/PAGE (10% gel, samples reduced) of heat-solubilized porcine ZP treated with purified caprine acrosin

Lanes 1 and 2, 75 μg of ZP, no acrosin, incubated for 0 h (lane 1) or 24 h (lane 2); lanes 3–8, 50 μg of ZP, 3 μg of acrosin, incubated for 0 h (lane 3), 1 h (lane 4), 3 h (lane 5), 5 h (lane 6), 10 h (lane 7) or 24 h (lane 8); lanes 9 and 10, no ZP, 3 μg of acrosin, incubated for 0 h (lane 9) or 24 h (lane 10).

Fig. 5. Two-dimensional PAGE of 200 μg of heat-solubilized porcine ZP treated for 0 (a) and 24 h (b) with 15 μg of purified caprine acrosin

Abbreviation: I.e.f., isoelectric focusing.

acrosin is a two-chain molecule wherein the chains are joined by disulphide bonding. The larger of the polypeptide chains migrated with an M_r of 40000 on SDS/PAGE. By h.p.l.c. in 6 M-guanidinium chloride on a calibrated gel-filtration column, the apparent M_r of the smaller polypeptide was 3700 ± 100 . These results are in excellent agreement with those for porcine acrosin, which is composed of a 37000- M_r heavy chain and a 4200- M_r light chain joined by two interchain disulphide bonds [9].

Purified porcine proacrosin (55000 M_r) autoactivates at alkaline pH to produce active acrosin (reviewed in [1]). At least three acrosins which differ in M_r are formed sequentially by autoactivation of purified porcine proacrosin, and two of these acrosins, designated m_α - and m_β -, have been isolated and partially characterized [2,3]. The nomenclature used for the acrosins produced during autoactivation of purified 55000- M_r porcine proacrosin is not applicable to all species, because multiple proacrosins exist in species such as the guinea pig [39], human [40], hamster [18] and rabbit [41]. In addition, since proacrosin activation *in vivo* may occur by a mechanism other than autoactivation [42,43], the structure of acrosin purified directly from extracts of spermatozoa, in which proacrosin activation occurred under undefined conditions, may differ from the structures of acrosins derived from autoactivation of purified proacrosin. Thus we are unable to specify whether the caprine acrosin described herein is the same structurally as acrosins produced by autoactivation. Additional studies will be necessary to determine the structural relations between the known proacrosins and acrosins.

Accurate estimation of the sizes of acrosin-system components is necessary for understanding acrosin structure and the mechanism of its formation from proacrosin. Unfortunately, some past estimates of acrosin size were unreliable because acrosin is a glycoprotein and thus may migrate anomalously in gel filtration [44], or because inadequate attention was paid to the oxidation state of the acrosin samples and/or standards. The apparent M_r values reported here were determined by using methods and conditions which minimize possible anomalies attributable to the enzyme's glycosylation and disulphide bonding.

Amino acid sequencing and amino acid composition of purified caprine acrosin light chain showed that it is an octadecapeptide. The presence of glucosamine indicated that the peptide is glycosylated. Porcine acrosin light chain is a glycopeptide; the carbohydrate moiety is attached to an asparagine residue at position 3 of the peptide [9]. The glycosylated asparagine residue was not sequencible by automated Edman degradation [9]. Since the third residue of caprine acrosin light chain was also not sequencible by automated Edman degradation, and amino acid analysis indicated presence of carbohydrate and four aspartic acid/asparagine residues per light chain, an asparagine residue was assigned to that position. The resultant amino acid sequence of caprine acrosin light chain exhibits 78 % positional identity with that of porcine acrosin [9]. However, caprine acrosin light chain is five residues shorter than porcine acrosin light chain. It is possible that the caprine acrosin described here autolysed after its initial formation from proacrosin, resulting in removal of a pentapeptide from the C-terminus of the light chain. The observation that

arginine is the C-terminal residue of caprine acrosin light chain is consistent with this hypothesis.

Acrosin shows sequence similarity to the trypsin family of serine proteinases; 77 % of the positions which are typically conserved and all (100 %) of the positions which are invariable in serine proteinases [45] are conserved in the N-terminal 52 residues of porcine acrosin [10]. In comparison, the N-terminal amino acid sequence of caprine acrosin heavy chain exhibits 70 % positional identity with that of porcine acrosin, and 73 % of typically conserved and all of the four invariable positions in serine proteinases are conserved in the N-terminal 37 residues of caprine acrosin.

Divergence rates of orthologous sequences vary depending on the protein. It seems reasonable to speculate that the sequence divergence rate of proteins with species-specific function would be unusually rapid, owing to the fact that inter-species sequence differences would be required to produce the species differences in function. Overall, caprine and porcine acrosins differ in 27 % of the 55 positions compared thus far. By using a divergence time for the caprine and porcine lineages of 50 million years ago (determined by comparing orthologous globin sequences [46]), one can calculate that acrosin primary structure is diverging at a rate of 1.8 million years per 1 % of change in sequence (i.e. unit evolutionary period for acrosin = 1.8 million years). By comparison, the unit evolutionary period of trypsinogen is 6 million years, and those of carbonic anhydrase, ribonuclease, and lactalbumin, proteins whose functions are not species-specific, have unit evolutionary periods of approx. 2 million years [47]. Thus, in this initial comparison, the sequence divergence rate of acrosin is not greater than those of proteins without species-specific function. Since binding of spermatozoa to the ZP is relatively species-specific [23], and recent evidence implicates proacrosin and/or acrosin in this event [48,49], it will be necessary to compare the sequences of the putative ZP binding domains of acrosin before final conclusions about the relation between sequence divergence rates and species specificity of function can be made. Comparison of complete proacrosin sequences from several species will ensure that the portion(s) of the proacrosin and acrosin molecules potentially involved in species-specific function are being compared.

Previous studies testing the effects of purified acrosin on ZP morphology showed that the enzyme could digest the ZP surrounding eggs from some heterologous species, and was unable to digest the homologous ZP [50]. The conflict presented by these results was reconciled by the observation that acrosin could effect limited proteolysis of the ZP without affecting the morphology of the envelope. Brown [51] showed that purified ovine and porcine acrosins had no effect on ovine ZP morphology, but rapidly hydrolysed ovine ZP components having M_r values of greater than 50000, leaving a 46000- M_r component intact. Similarly, porcine acrosin preferentially hydrolyses the 90 and 65 kDa components of isolated porcine ZP [19–21]. Purified caprine acrosin also preferentially hydrolysed the 90 and 65 kDa components of heat-solubilized porcine ZP. Thus we detected no species-specificity in the proteolytic action of acrosin on porcine ZP glycoproteins. Our results do not preclude the possibility of species-specificity in the early events of acrosin action on the ZP, since the shortest treatment we studied was 1 h.

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REFERENCES

- Parrish, R. F. & Polakoski, K. L. (1979) *Int. J. Biochem.* **10**, 391–395
- Parrish, R. F. & Polakoski, K. L. (1977) *Biol. Reprod.* **17**, 417–422
- Parrish, R. F. & Polakoski, K. L. (1978) *J. Biol. Chem.* **253**, 8428–8432
- Muller-Esterl, W., Kupfer, S. & Fritz, H. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1811–1821
- Brown, C. R. & Hartree, E. F. (1978) *Biochem. J.* **175**, 227–238
- Brown, C. R. (1983) *J. Reprod. Fertil.* **69**, 289–295
- Mukerji, S. K. & Meizel, S. (1979) *J. Biol. Chem.* **254**, 11721–11728
- Muller-Esterl, W. & Fritz, H. (1981) *Methods Enzymol.* **80**, 621–632
- Fock-Nuzel, R., Lottspeich, F., Henschen, A. & Muller-Esterl, W. (1984) *Eur. J. Biochem.* **141**, 441–446
- Fock-Nuzel, R., Lottspeich, F., Henschen, A., Muller-Esterl, W. & Fritz, H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1823–1828
- Lui, C. W. & Meizel, S. (1979) *J. Exp. Zool.* **207**, 173–186
- Meizel, S. (1984) *Biol. Rev.* **59**, 125–157
- Green, D. P. L. (1978) *J. Cell Sci.* **32**, 153–164
- Perreault, S. D., Zirkin, B. R. & Rogers, B. J. (1982) *Biol. Reprod.* **26**, 343–351
- Huang, T. T. F., Hardy, D., Yanagimachi, H., Teuscher, C., Tung, K., Wild, G. & Yanagimachi, R. (1985) *Biol. Reprod.* **32**, 451–462
- Saling, P. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6231–6235
- Stambaugh, R. & Buckley, J. (1969) *J. Reprod. Fertil.* **19**, 423–432
- Meizel, S. & Mukerji, S. K. (1976) *Biol. Reprod.* **14**, 444–450
- Urch, U. A., Wardrip, N. J. & Hedrick, J. L. (1985) *J. Exp. Zool.* **233**, 479–483
- Brown, C. R. & Cheng, W. T. K. (1985) *J. Reprod. Fertil.* **74**, 257–260
- Dunbar, B. S., Dudkiewicz, A. B. & Bundman, D. S. (1985) *Biol. Reprod.* **32**, 619–630
- Hedrick, J. L. & Wardrip, N. J. (1987) *Dev. Biol.* **121**, 478–488
- Yanagimachi, R. (1977) in *Immunobiology of Gametes* (Edidin, M. & Johnson, H., eds.), pp. 255–295, Cambridge University Press, Cambridge
- Yanagimachi, R. (1981) in *Fertilization and Embryonic Development in Vitro* (Mastroianni, L. & Biggers, J. D., eds.), pp. 81–182, Plenum Press, New York
- O'Rand, M. G. (1986) in *The Molecular and Cellular Biology of Fertilization* (Hedrick, J. L., ed.), pp. 383–393, Plenum Press, New York
- Schoots, A. F. M. & Hedrick, J. L. (1983) *J. Cell Biol.* **97**, 30A
- Hedrick, J. L. & Wardrip, N. J. (1986) *Anal. Biochem.* **157**, 63–70
- Carroll, E. J. & Hedrick, J. L. (1974) *Dev. Biol.* **38**, 1–13
- Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta.* **16**, 570–575
- Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley and Sons, New York
- Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) *J. Biol. Chem.* **193**, 265–175
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Ui, N. (1981) *J. Chromatogr.* **215**, 289–294
- Edman, P. & Henschen, A. (1975) in *Protein Sequence Determination* (Needleman, S. B., ed.), 2nd edn., pp. 232–279, Springer Verlag, New York
- Hunkapiller, M. W. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 486–493
- Bhown, A. S., Mole, A., Weissinger, A. & Bennett, J. C. (1978) *J. Chromatogr.* **148**, 532–535
- Hardy, D. M., Tung, K. S. K. & Wild, G. C. (1987) *Biol. Reprod.* **37**, 189–199
- Tobias, P. S. & Schumacher, G. F. B. (1975) *Biochem. Biophys. Res. Commun.* **74**, 434–439
- Meizel, S. & Mukerji, S. K. (1975) *Biol. Reprod.* **13**, 83–93
- McRorie, R. A., Turner, R. B., Bradford, M. M. & Williams, W. L. (1976) *Biochem. Biophys. Res. Commun.* **71**, 492–498
- Srivastava, P. N. & Ninjoor, V. (1982) *Biochem. Biophys. Res. Commun.* **109**, 63–69
- Beeley, J. G. (1985) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Burdon, R. H. & van Knippenberg, P. H., eds.), vol. 16 (Glycoprotein and Proteoglycan Techniques), pp. 63–99, Elsevier Science Publishers B. V., Amsterdam
- Young, C. L., Barker, W. C., Tomaselli, C. M. & Dayhoff, M. O. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed.), vol. 5, suppl. 3, pp. 73–93, National Biomedical Research Foundation, Washington
- Goodman, M. (1977) in *Molecular Evolution* (Ayala, F. J., ed.) pp. 141–159, Sinauer Associates, Sunderland, MA
- Wilson, A. C., Carlson, S. S. & White, T. J. (1977) *Annu. Rev. Biochem.* **46**, 573–639
- Jones, C. R. & Brown, C. R. (1987) *Exp. Cell Res.* **171**, 503–508
- Toepfer-Petersen, E. & Henschen, A. (1987) *FEBS Lett.* **226**, 38–42
- Brown, C. R. (1982) *J. Reprod. Fertil.* **64**, 457–462
- Brown, C. R. (1986) *J. Reprod. Fertil.* **77**, 411–417