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Site-directed Mutagenesis of Rabbit Proacrosin

IDENTIFICATION OF RESIDUES INVOLVED IN ZONA PELLUCIDA BINDING*

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The mammalian acrosomal sperm protease proacrosin plays a role in fertilization by proteolysis of the oocyte's outer investments. In addition to its serine protease activity, acrosin from several species is known to have binding activity for the zona pellucida, and this action may serve to anchor sperm during zona penetration. In this study, proacrosin was purified from acid extracts of rabbit sperm and shown to bind to homologous zona pellucida using an *in vitro* assay. Measurement of this binding activity indicated a high affinity saturable interaction with a $K_D = 1.4 \times 10^{-8}$ M.

Using cDNAs obtained from previously cloned and sequenced rabbit proacrosin and a splice variant that encodes a shorter form of acrosin (Richardson, R. T., and O'Rand, M. G. (1994) *Biochim. Biophys. Acta* 1219, 215–218), constructs of various sizes were produced using polymerase chain reaction and expressed as recombinant proteins. In the same *in vitro* zona binding assay, a construct representing residues 1–279 of rabbit proacrosin was found to bind to zona with a high affinity similar to that of native proacrosin, $K_D = 2.1 \times 10^{-8}$ M. By making smaller recombinant fragments and assaying them for zona binding activity, the location of the binding site was mapped to residues 47–94. Protein modeling of rabbit proacrosin using chymotrypsinogen A as a three-dimensional model indicated that an exposed loop Asp^{35} to His^{40} in chymotrypsinogen A is extended with an additional five amino acid residues in rabbit proacrosin from Ile^{43} to His^{53} containing arginine residues Arg^{47}, Arg^{50} and Arg^{51}. Site-directed mutagenesis of arginine residues Arg^{50} and Arg^{51} to alanine produced a recombinant without significant zona binding activity. These results are consistent with the hypothesis that rabbit proacrosin contains a specific zona pellucida binding site and that the loop containing arginine residues 50 and 51 is critical for zona binding activity.

The unique spermatozoon-derived serine protease, acrosin (EC 3.4.21.10), serves multiple roles in fertilization (2, 3), assisting in the spermatozoon's penetration of the oocyte's outer covering, the zona pellucida (ZP)[1] (4). Initial sperm-ZP recognition and binding, followed by the acrosome reaction and secondary sperm binding to the ZP is the general sequence of events leading to membrane fusion and fertilization in mammals. In intact spermatozoa, acrosin occurs as a single chain molecule in the inactive precursor form of proacrosin. After activation and autocatalytic cleavage at amino acid 23, a two-chain active acrosin molecule is formed with a light and heavy chain that has several different molecular weights, depending upon the degree of C-terminal heavy chain autoproteolysis (3, 5). In several species proacrosin and acrosin have been shown to bind to the homologous ZP (6–9), but this binding is not restricted by species specificity (9). In fact, boar proacrosin and acrosin are known to bind various polysaccharides and particularly to those containing terminal sulfates (7, 10). Topfer-Petersen *et al.* (11) suggested that the zona binding activity of boar proacrosin is associated with a 15-kDa autocatalytically generated peptide from the N terminus. More recently, Jansen *et al.* (12) assayed recombinant fragments of boar proacrosin and compared their zona binding ability with that of the native acrosin. They found that a construct containing approximately 30 kDa of the N-terminal sequence had binding activity equal to that of the native molecule. Consequently, interaction of proacrosin and acrosin with the ZP may serve at least two purposes. The first may be to anchor the enzyme to its natural substrate. The second may be to regulate conversion of proacrosin to enzymatically active acrosin (13, 14) and up- or down-regulate the activity of the active enzyme (15).

Similar to proacrosin from several other species, rabbit proacrosin exhibits a M_r of approximately 53,000–55,000, which is usually seen as a doublet on Western blots (16). Upon activation, rabbit proacrosin is converted into the two-chain, 34-kDa mature form (17). As described previously, after the zona pellucida induces the acrosome reaction of rabbit spermatozoa, acrosin is localized on the outer surface of the cell in the equatorial and postacrosomal regions (16). It is on this surface that acrosin may serve as a secondary binding site for the spermatozoon (16).

The present study was designed to address the question of whether or not rabbit proacrosin contains a zona binding site(s) that could function in secondary binding. To determine the binding site(s) for zona pellucida on the rabbit proacrosin molecule, constructs of various sizes were produced and expressed as recombinant forms of proacrosin, and their binding affinities were compared with that of purified native proacrosin.

EXPERIMENTAL PROCEDURES

Materials—Frozen rabbit ovaries were obtained from Pel-Freez (Rogers, AR). All other chemicals were of the highest available quality.

Protein Determinations—All protein concentrations were determined in duplicate using the Micro BCA assay (Pierce), using bovine serum albumin as the standard.

Production and Expression of Recombinants—cDNAs expressed as proteins (see Fig. 2) were generated by polymerase chain reaction from λ gt 11 clones of either proacrosin or the shorter splice variant of acrosin (1) using primers designed to produce 5′ *Bam*HI and 3′ *Kpn*I restriction sites to facilitate directional cloning into the expression vector pQE-30

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[1] The abbreviations used are: ZP, zona pellucida; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HSRZ, heat-solubilized rabbit zona pellucida; RPA, rabbit proacrosin; shRPA, shorter splice variant of RPA; BAPNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide.

TABLE I
Purification of rabbit proacrosin

Step	Fraction	Protein	BAPNA activity	Specific activity	Purification	Yield
		mg	*milliunits*	*milliunits/mg*	*-fold*	*%*
1	Acid extract	20.4	NA[a]	NA	NA	NA
2	Sephadex G-100	1.68	1350	804	NA	100
3	Prep electrophoresis	0.096	961	10,010	12.5	71

[a] NA, not applicable.

(Qiagen Co., Chatsworth, CA). All clones were sequenced to confirm reading frame and sequence. Expression and purification of recombinants were performed as described previously (18).

Preparation of Antiserum and Enzyme-linked Immunosorbent Assays—Polyclonal antiserum directed against amino acids 1–279 of rabbit proacrosin (mouse anti-Ace) was prepared by immunizing BALB/c mice with Ace fusion protein. An initial injection of 100 μl of Ace (20 μg/ml, 1:1 with complete adjuvant) was followed by two boosters 3 and 5 weeks later, consisting of 100 μl of Ace (20 μg/ml), 1:1 with incomplete adjuvant. The mice were bled for 5 weeks, every 7–10 days. High titers against both Ace fusion protein and purified proacrosin were observed on both Western blots and in enzyme-linked immunosorbent assay (data not shown).

Electrophoresis and Western Blotting—SDS-PAGE was performed according to the method of Laemmli (19). Samples for analysis were reduced by adding β-mercaptoethanol and boiling for 3 min. Western blotting and antibody staining of blots were performed as described previously (16).

Proacrosin Purification and Assay—Ejaculated rabbit spermatozoa were collected using an artificial vagina. To prepare the acid extract, 21 ejaculates containing 1.8×10^9 spermatozoa were washed by centrifugation three times in 20 volumes of phosphate-buffered saline, pH 7.2 (PBS) with 1 mM *p*-aminobenzamidine. The sperm were suspended in 20 ml of 0.3 M acetic acid, 0.05 M sodium chloride, the pH was adjusted to 3.0, and the suspension was sonicated and stirred overnight at 4 °C. The suspension was centrifuged at 20,000 $\times$ *g* for 30 min at 4 °C, and the supernatant was Speedvac (Savat, Inc.) concentrated to 3 ml, dialyzed overnight against 1 mM HCl, and centrifuged again at 20,000 $\times$ *g* for 30 min. This acid extract was loaded onto a Sephadex-G-100 (Pharmacia Biotech Inc.) column (1 $\times$ 13 inches). After gel filtration in 1 mM HCl, individual 1.25-ml fractions were assayed for arginine amidase activity using the BAPNA assay (20), scaled down for use in microwell plates. Briefly, 170 μl of 0.2 M triethanolamine hydrochloride buffer (pH 7.8) with 2 mM $CaCl_2$, 100 μl of 1.15 mM *N*-benzoyl-L-arginine *p*-nitroanilide (Sigma) in H_2O, and 30 μl of test solution were added to each well. Absorbance at 405 nm was measured over 1 h, and any fraction with ΔAbs >0.5 was pooled for the next purification step. The pooled sample was Speedvac-concentrated 20 times to 2 ml, mixed with 1 ml of 2 $\times$ Laemmli sample buffer without β-mercaptoethanol but including 1 mM benzamidine, and applied to a Bio-Rad model 491 Prep Cell apparatus. Electrophoresis was performed for 14 h at 9.0 constant watts using the 37-mm inside diameter cell filled to 7.0 cm with 10% SDS-acrylamide gel and 1-cm stacking gel, according to the manufacturer's directions using Laemmli running buffer with 1 mM benzamidine added. Fractions eluted from the cell were checked in an enzyme-linked immunosorbent assay using mouse anti-Ace to detect the presence of proacrosin, followed by SDS-PAGE of the positive fractions, Western blotting, and detection with anti-Ace on the blot to confirm the molecular weight. The proacrosin-containing fractions (M_r 53,000–55,000) were pooled and dialyzed against 1 mM HCl and tested for activity in a standard BAPNA assay (20), wherein one unit of acrosin activity is defined as ΔA = 3.3/min.

Zona Pellucida Isolation, Labeling, and Quantitative Zona Binding Assay—The preparation and heat solubilization of zonae pellucidae from frozen rabbit ovaries has been described in detail previously (21, 22). Heat-solubilized rabbit zona pellucida (HSRZ) was ^{125}I-labeled by adding 150 μl of HSRZ solution (100 μg/ml) and 100 μCi of ^{125}I-labeled NaI to a glass tube previously coated with Iodagen reagent (Pierce) according to the manufacturer's protocol. The tube was gently rocked for 30 min, and the reaction was stopped with 10 μl of KI solution (2.5 M) followed by purification using a Bio-Spin 6 chromatography column (Bio-Rad).

The *in vitro* zona binding assay was a modification of the method employed by Jones (7). In this assay, specific quantities of either proacrosin or the recombinant proteins were immobilized onto a nitrocellulose membrane (0.45 μm) using a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was then blocked in a solution of 5% bovine serum albumin in PBS for 30 min. After draining off the blocking solution, the membrane was put in 2 μg/ml ^{125}I-HSRZ (approximately 10^6 cpm/μg) in PBS with 1% bovine serum albumin for 1 h followed by washing two times in PBS for 10 min each. All incubations were at room temperature. Finally, the blot was cut into 1-cm squares, with each square containing only a single dot of protein. The squares were then counted in an Pharmacia γ counter. In the time course experiment (see Fig. 3), instead of a 1-h incubation, individual 1-cm squares were removed at specific time points before counting. When assessing saturability of binding (see Fig. 5*A*), the dot-blotted proteins were incubated on separate 1-cm squares in varying ^{125}I-HSRZ concentrations. To demonstrate competition for binding by unlabeled HSRZ, the individual squares were put into the 2 μg/ml ^{125}I-HSRZ in PBS with 1% bovine serum albumin except that additional unlabeled HSRZ was added in increasing amounts.

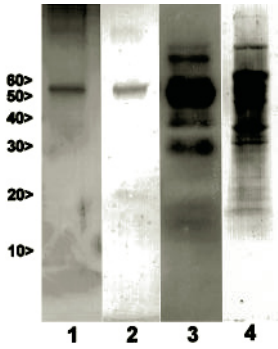


FIG. 1. **10–20% gradient minigel of purified and partially purified rabbit proacrosin.** *Lane 1*, silver-stained gel of purified rabbit proacrosin (1 μg). *Lane 2*, purified rabbit proacrosin (0.1 μg), Western blot-detected using mouse anti-Ace. *Lane 3*, acid extract after gel filtration (15 μg), probed with ^{125}I-HSRZ (autoradiograph of Western blot). *Lane 4*, acid extract after gel filtration (15 μg), stained with Amido Black. Molecular masses on *left* are in kDa.

Site-directed Mutagenesis—Mutagenesis was performed using the Altered Sites II *in vitro* mutagenesis system (Promega Corp., Madison, WI) according to the manufacturer's recommended protocol. Briefly, Ace A cDNA insert was excised from pQE-30 using *Bam*HI and *Kpn*I (Boehringer Mannheim) and cloned into these sites in the p-ALTER-1 vector. Single-stranded DNA template for mutagenesis was prepared using helper phage R408 in conjunction with JM 109 bacteria. In two separate reactions, the ampicillin repair oligonucleotide and one each of the following phosphorylated mutagenic oligonucleotides were annealed to the Ace A/p-ALTER single-stranded DNA: 1 mut A, 5′CGCCCCGCAACAATGCCGCATACCACGCGTGCGG3′; 2 mut A, 5′GCCCACTGCTTCAACGCGGCACAGAAAGTCTATGAG3′.

After second strand synthesis and ligation, the DNA was transformed into the repair minus *Escherichia coli* strain BHM 71–18 mut-S, and positive clones were selected by growth on ampicillin, followed by sequencing to confirm the mutation.

Statistics—Statistical analysis was performed by Student's *t* test using SigmaStat software (Jandel Scientific).

Three-dimensional Protein Modeling—Three-dimensional protein modeling of proacrosin was run on the Swiss-Model version of ProMod (23) using chymotrypsinogen A (2CGA.pdb) as the reference structure and α-chymotrypsin as the three-dimensional matched structure (1ABC.pdb). The resulting three-dimensional proacrosin image was prepared as a kinemage using PERKIN and displayed by MAGE (24). Fig. 8 was prepared by displaying and printing the proacrosin pdb file in RasMol2.[2]

[2] For further information, please contact Roger Sayle at ras32425@ggr.co.uk.

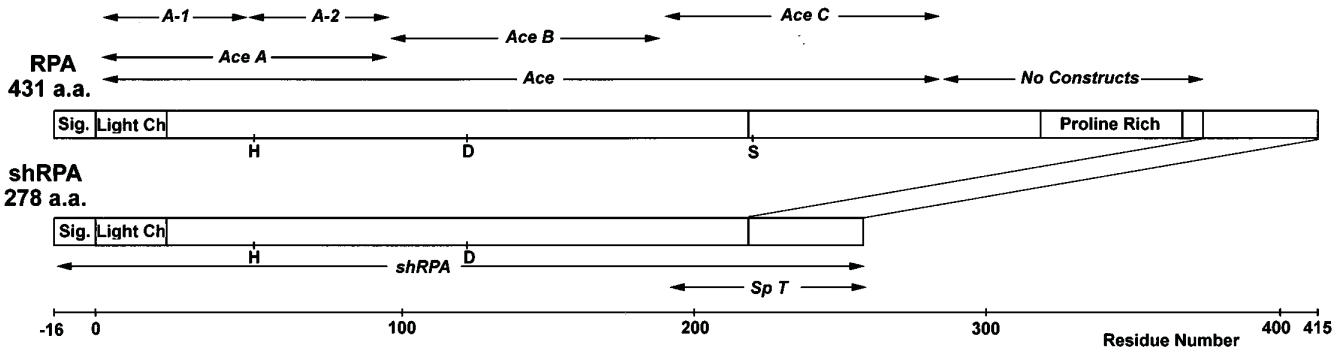


FIG. 2. **Diagram of the rabbit proacrosin system showing the various recombinants prepared to test for zona binding.** The *horizontal bars* depict RPA with 431 residues and shRPA, which is identical to RPA except for the omission of residues 220–376 in the C-terminal region, indicated by the *gray area*. Sequences of the various recombinants, shown as *lines* with *arrowheads* are shown in Table II. Note that all residues in the shRPA sequence are represented by the constructs, and all of the RPA sequence except residues 279–376 is represented.

TABLE II
Sequences of recombinants shown in Fig. 2

Designation	Residues	Parent sequence
Ace A-1	1–46	RPA
Ace A-2	47–94	RPA
Ace A	1–94	RPA
Ace B	94–186	RPA
Ace C	187–279	RPA
Ace	1–279	RPA
shRPA	1–278	shRPA
SpT	187–278	shRPA

RESULTS

Purification of Rabbit Proacrosin—In a three-step method (Table I), proacrosin was purified from rabbit sperm to a specific activity of 10,010 milliunits/mg. Since ejaculates typically contain endogenous protease inhibitors, amidase activity on the crude acid extract could not be determined, and the degree of purification could only be calculated for steps II and III. SDS-PAGE, Western blotting, and Amido Black staining of the acid extract after gel filtration indicated four bands in addition to a broad band at M_r 48,000–55,000 (Fig. 1, *lane 4*). When the acid extract was probed with ^{125}I-heat-solubilized rabbit zona, a strong band was seen at 50–55 kDa (Fig. 1, *lane 3*), corresponding to the M_r of rabbit proacrosin under reducing conditions (1). After purification, rabbit proacrosin appeared as a single band of 53–55 kDa on a silver-stained gel (Fig. 1, *lane 1*) and on a Western blot when probed with mouse anti-Ace (Fig. 1, *lane 2*), indicating that a purified preparation of proacrosin had been obtained.

Assessment of the Zona Pellucida—The isolated zona pellucida "ghosts" were microscopically checked for purity and lack of cellular debris before solubilization. ^{125}I-Heat-solubilized zona pellucida after SDS-PAGE (7%) under reducing conditions gave a broad radioactive band from 75 to 105 kDa (data not shown), which was identical to a previous study that assigned the combined rabbit zona pellucida components a M_r range between 70,000 and 110,000 (25).

Production of Recombinants—Recombinants were selectively prepared and tested for zona binding activity. Fig. 2 and Table II diagram the positions of the various constructs relative to rabbit proacrosin (RPA) and the shorter splice variant, shRPA.

Specificity of Proacrosin-Zona Pellucida Binding—Using the quantitative assay, binding of proacrosin and Ace fusion protein to ^{125}I-HSRZ very nearly paralleled each other and were complete at about 40 min of incubation (Fig. 3). As shown in Fig. 4, purified proacrosin and the recombinants Ace and Ace A bound ^{125}I-HSRZ in a linear fashion over the range of 2.5–20 pmol. Under the assay conditions, binding of ^{125}I-HSRZ to proacrosin reached saturation at about 2 μg/ml. Binding of

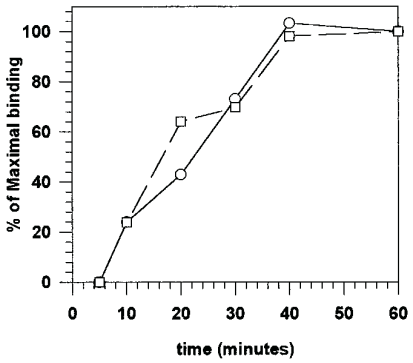


FIG. 3. **Time course of ^{125}I-heat-solubilized rabbit zona pellucida binding to rabbit proacrosin and Ace fusion protein.** In the standard dot blot assay, 10 pmol of either proacrosin (○) or Ace fusion protein (□) was applied to nitrocellulose followed by incubation in 2 μg/ml ^{125}I-HSRZ. Incubations were terminated at specific time points by removal from the zona solution and washing two times for 10 min each in PBS. Maximal binding was defined as ^{125}I-HSRZ bound at 60 min.

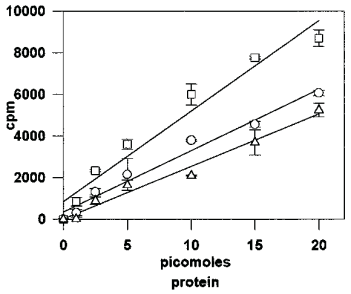


FIG. 4. **Binding of ^{125}I-heat-solubilized rabbit zona pellucida to increasing quantities of acrosin, Ace, and Ace A is linear.** In the standard dot blot assay, increasing amounts of acrosin (□), Ace (○), and Ace A (Δ) were applied to nitrocellulose, followed by incubation in 2 μg/ml ^{125}I-HSRZ. Each data point is the mean of triplicate measurements, the experiment was run in duplicate, and the results shown are representative of a typical data set.

^{125}I-HSRZ to proacrosin was saturable, and analysis by Scatchard plot indicated a straight line function with a K_D for proacrosin of 1.4×10^{-8} M (Fig. 5, *A* and *B*), assuming a molecular mass for rabbit zona of 90 kDa, which corresponds to the average molecular mass exhibited by ZP on SDS-PAGE. In a similar assay using boar sperm and pig zona, a K_D of 1.2×10^{-8} M was obtained (7). K_D values were also determined for the recombinants Ace and Ace A. Similar to proacrosin, these recombinants also bound ^{125}I-HSRZ with a high affinity as shown in Table III. The binding of ^{125}I-HSRZ to both proacrosin and Ace A could be inhibited to background levels by competition with unlabeled HSRZ (data not shown).

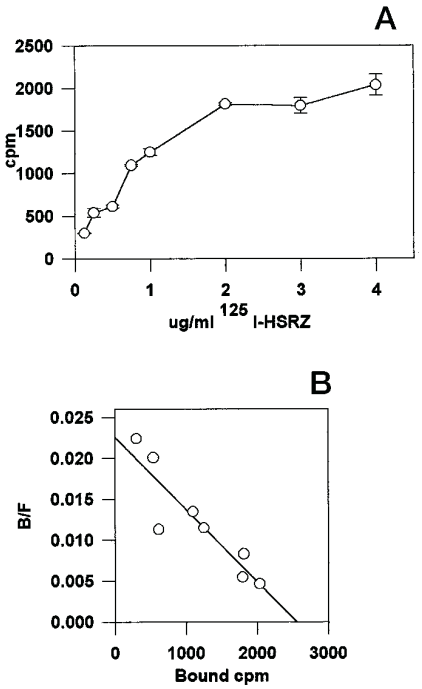


FIG. 5. **Binding of ^{125}I-heat-solubilized rabbit zona pellucida to rabbit proacrosin is saturable.** *A*, in the standard dot blot assay, 10 pmol of purified rabbit proacrosin applied to nitrocellulose was incubated with increasing concentrations of ^{125}I-HSRZ. Each data point represents the mean of duplicate measurements. *B*, Scatchard analysis for proacrosin was performed on saturation data from *A*. *B* represents a typical analysis, and the K_D values are given in Table III.

TABLE III
Dissociation constants of rabbit proacrosin, Ace, and Ace A recombinants

Protein	K_D[a]
	mol/liter
Proacrosin[b]	1.40×10^{-8}
Ace fusion protein[b]	2.12×10^{-8}
Ace A fusion protein[b]	2.43×10^{-8}

[a] Based on rabbit zona having a molecular mass of 90 kDa.
[b] Average of two separate experiments.

Binding of ^{125}I-HSRZ to Rabbit Proacrosin Recombinants—Western blotting and dot blot assays of six rabbit proacrosin recombinants indicated that ZP bound strongly to Ace, Ace A, and shRPA but only slightly above background to Ace B, Ace C, and SpT (Figs. 6*A*, and 7*A*). Preincubation of Western blots of acrosin recombinants with fucoidan completely blocked ZP binding activity (Fig. 6*B*). The recombinant construct Ace retained 84.1% of native proacrosin's ^{125}I-HSRZ binding activity (Fig. 7*A*), an amount not significantly different from native proacrosin ($p > 0.01$).

Since the constructs Ace, Ace A, and shRPA overlap (Fig. 2, Table II) and have identical amino acid sequences at positions 1–94, the major proacrosin binding site is most likely within these residues and not in the other sequences tested. To map the ZP binding activity within the Ace A construct, the sequence was divided into an N-terminal half, Ace A-1 (residues 1–46), and a C-terminal half, Ace A-2 (residues 47–94), as shown in Fig. 2 and Table II. The binding of ^{125}I-HSRZ to the N-terminal construct, Ace A-1, was only slightly above background levels (Fig. 7, *A* and *B*) and significantly different from Ace A ($p < 0.00063$). In contrast, the binding of ^{125}I-HSRZ to the C-terminal construct, Ace A-2, was not significantly different from Ace A and retained 50.4% of native proacrosin's ability to bind ^{125}I-HSRZ and 78% of Ace A's ability to bind ^{125}I-HSRZ.

Three-dimensional Protein Modeling—An examination of the amino acid sequence of Ace A-2 (Fig. 7*B*) for arginine and lysine residues that might act as charge-charge interaction sites with the zona pellucida indicated that there were three arginine residues at positions 47, 50, and 51, two lysine residues at positions 75 and 77, and an additional arginine residue at position 82. Three-dimensional protein modeling of proacrosin amino acids 10–252 in the Swiss-Model version of ProMod (23) with chymotrypsinogen A amino acids 1–223 as the reference structure indicated that the Ace A-2 portion of the proacrosin molecule mimicked the chymotrypsinogen A structure in this region. In this region, chymotrypsinogen A has two loops exposed to the exterior of the molecule, one from Asp^{35} to His^{40} and one from Gly^{59} to Asp^{64}. Chymotrypsinogen A loop Asp^{35} to His^{40} in rabbit proacrosin is extended with an additional five amino acid residues from Ile^{43} to His^{53}, containing the three arginine residues at positions 47, 50, and 51, while chymotrypsinogen A loop Gly^{59} to Asp^{64} contains rabbit proacrosin lysines 75 and 77 (Fig. 8*A*). Consequently, two site-directed mutants were produced (Fig. 7, *A* and *B*, and Fig. 8, *B* and *C*), one with Arg^{50} → Ala and Arg^{51} → Ala (1 mut A), which would be in loop Ile^{43} to His^{53} (Fig. 8*B*), and a second mutant with Asn^{74} → Ala and Lys^{75} → Ala (2 mut A), which would be in chymotrypsinogen A loop Gly^{59} to Asp^{64} (proacrosin loop Phe^{72} to Lys^{77}; Fig. 8*C*). Subsequent testing of their zona binding activity demonstrated that recombinant 1 mut A retained only 12% of the binding activity of its parent sequence Ace A, whereas 2 mut A activity was not significantly affected (Fig. 7, *A* and *B*). Three-dimensional modeling of 1 mut A indicated a change in the conformation of the mutated loop (Ile^{43} to His^{53}) but did not indicate any gross changes in the overall structure of the molecule. Thus, the zona binding activity of the Ace A-2 construct occurs within a region that contains the loop with Arg^{50} and Arg^{51}.

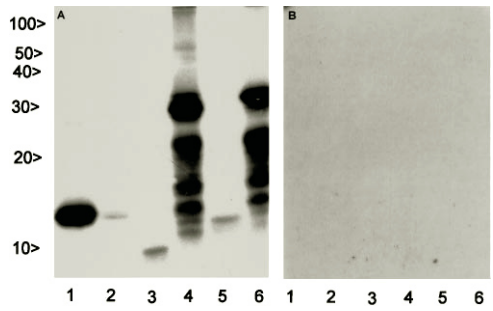
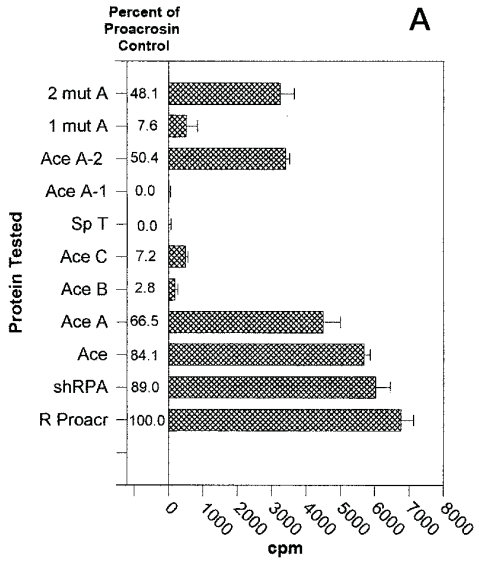


FIG. 6. **^{125}I-Heat-solubilized rabbit zona pellucida binds to specific acrosin recombinants on a Western blot.** 10–20% gradient SDS-PAGE and Western blotting of 100 pmol of rabbit proacrosin recombinant proteins. Proteins were loaded as follows: Ace A (*lane 1*), Ace B (*lane 2*), Ace C (*lane 3*), Ace (*lane 4*), SpT (*lane 5*) shRPA (*lane 6*). *A*, the blot was incubated in ^{125}I-HSRZ (2 μg/ml) for 1 h, washed, and autoradiographed. *B*, before incubation with zona, the blot was incubated in fucoidan (1 mg/ml) and washed. Molecular masses on *left* are in kDa.

DISCUSSION

This study has isolated enzymatically active RPA of high purity in a short, three-step process and demonstrated its ability to bind HSRZ with high affinity. Moreover, using recombinant proteins and site-directed mutagenesis, amino acids 47–94 have been identified as containing the major binding site for HSRZ. Within this region amino acids Arg^{50} and Arg^{51} play a key role in the binding activity. Systematic substitutions of single amino acids within the 47–94 sequence would be necessary to define all the critical residues. RPA binds to HSRZ with high affinity (1.4×10^{-8} M; Fig. 5*B*), which is comparable with values obtained in two previous studies measuring boar sperm proacrosin binding to heat-solubilized pig zona (2×10^{-8} M (10) and 1.2×10^{-8} M (7)). On Western blots (Fig. 6*A*) both proacrosin recombinants Ace and shRPA appear to have smaller M_r



B

Construct Sequence	Name	^{125}I-HSRZ bound,% control
KDNATCDGPC GLRFRQNPQG GFRVVGGQAA QQGAWPWMVS LQIFTPRNNR RYHACGGVLL NAHWVLTAAH CFNNKQKVYE WRMVFGAQEI EYGT	Ace A	100
KDNATCDGPC GLRFRQNPQG GFRVVGGQAA QQGAWPWMVS LQIFTP	Ace A-1	0
RNNR RYHACGGVLL NAHWVLTAAH CFNNKQKVYE WRMVFGAQEI EYGT	Ace A-2	78.0
KDNATCDGPC GLRFRQNPQG GFRVVGGQAA QQGAWPWMVS LQIFTPRNNA AYHACGGVLL NAHWVLTAAH CFNNKQKVYE WRMVFGAQEI EYGT	1 mut A	12.3
KDNATCDGPC GLRFRQNPQG GFRVVGGQAA QQGAWPWMVS LQIFTPRNNR RYHACGGVLL NAHWVLTAAH CFNAAQKVYE WRMVFGAQEI EYGT	2 mut A	81.9

FIG. 7. *A*, dot blot assay to compare zona binding with various proteins. 10 pmol of various recombinant proteins or purified rabbit proacrosin were applied to nitrocellulose (each done in triplicate), blocked, and incubated in ^{125}I-HSRZ (2 μg/ml) for 1 h and washed, and 1-cm squares were cut out and counted. Each data point is the mean of triplicate measurements, the experiment was run in triplicate, and the results shown are representative of a typical data set. *B*, sequences of constructs and mutants comparing ^{125}I-heat-solubilized rabbit zona pellucida binding. ^{125}I-HSRZ binding to the Ace A group of recombinants and mutants is compared based on binding to Ace A.

components, which also bind HSRZ. These bands were not seen on a protein-stained blot with the same protein loading (100 pmol) but were visible when stained with anti-Ace antiserum (data not shown). Since the recombinants carry a six-histidine tag at the N-terminal end for affinity purification, these fragments were each missing part of their C-terminus and were either the result of prematurely truncated mRNAs or more likely were incomplete translation products. Autoproteolysis did not cause the breakdown to smaller fragments, because neither recombinant showed enzymatic activity in the BAPNA assay. A previous study also reported that all acrosin forms, including the small autolytic fragments of M_r 12,000–18,000, bind ^{125}I-zona proteins on Western blots (3). Zona binding was blocked by excess fucoidan on Western blots (Fig. 6*B*), which is consistent with data obtained in the porcine system in which ^{125}I-zona binding to acrosin was inhibited by fucoidan and other sulfated polysaccharides (6, 7).

Topfer-Petersen *et al.* (11) demonstrated that the zona binding activity of boar acrosin was associated with a 15-kDa peptide containing amino acid residues 24 to approximately 150 from the N terminus, which includes a sequence with a high homology to RPA amino acids 1–94. In a study using recombinant boar proacrosin constructs, Jansen *et al.* (12) found that a fragment representing residues 3–275 bound ^{125}I-zona equally as well as the native molecule and that residues 102–179 bound heat-solubilized pig zona at levels near background. The 102–179 boar construct corresponds approximately to Ace B, which also bound ZP at very low levels. However, a construct with residues 3–179, similar in size to Ace A and Ace B combined, only retained about 20% ^{125}I-HSRZ and 40% ^{125}I-fucoidan binding activity. This might be explained by their use of the PRSET-T7 expression vector, which adds a protein tag of approximately 7.5 kDa to the expressed sequence that could easily disrupt normal folding and change exposed residues.

In the present study, it became apparent that bacterial expression of proteins containing constructs near the C-terminal region of proacrosin was limited. This region contains the proline-rich area that is found in all known acrosin sequences. Attempts to express these constructs were unsuccessful, including the use of various growth conditions and the use of at least six different bacterial strains. The part of the molecule that could not be tested as a recombinant included only 96 residues (Fig. 2 and Table II). However, the ^{125}I-HSRZ binding assay demonstrated a valid, saturable binding to native RPA as well as to the proacrosin recombinants. Recombinants Ace and Ace A had dissociation constants similar to native proacrosin (Table III), although they represent only 67 and 23%, respectively, of the total proacrosin molecule. Moreover, recombinant Ace essentially retained the ZP binding activity of the native proacrosin, although some additional binding activity could have been contributed by the native conformation of proacrosin. Clearly, both the ^{125}I-HSRZ binding assay and the Western blots (Figs. 6 and 7) demonstrated that ZP binding was restricted to Ace, Ace A, and Ace A-2, all of which contain amino acids 47–94. Interestingly, the light chain, containing three positively charged residues, did not have significant zona binding ability. In fact, pretreatment of Ace with mouse anti K20C (antiserum against the light chain peptide (1)) did not significantly reduce zona binding compared with mouse anti-Ace, which did block most binding (data not shown).

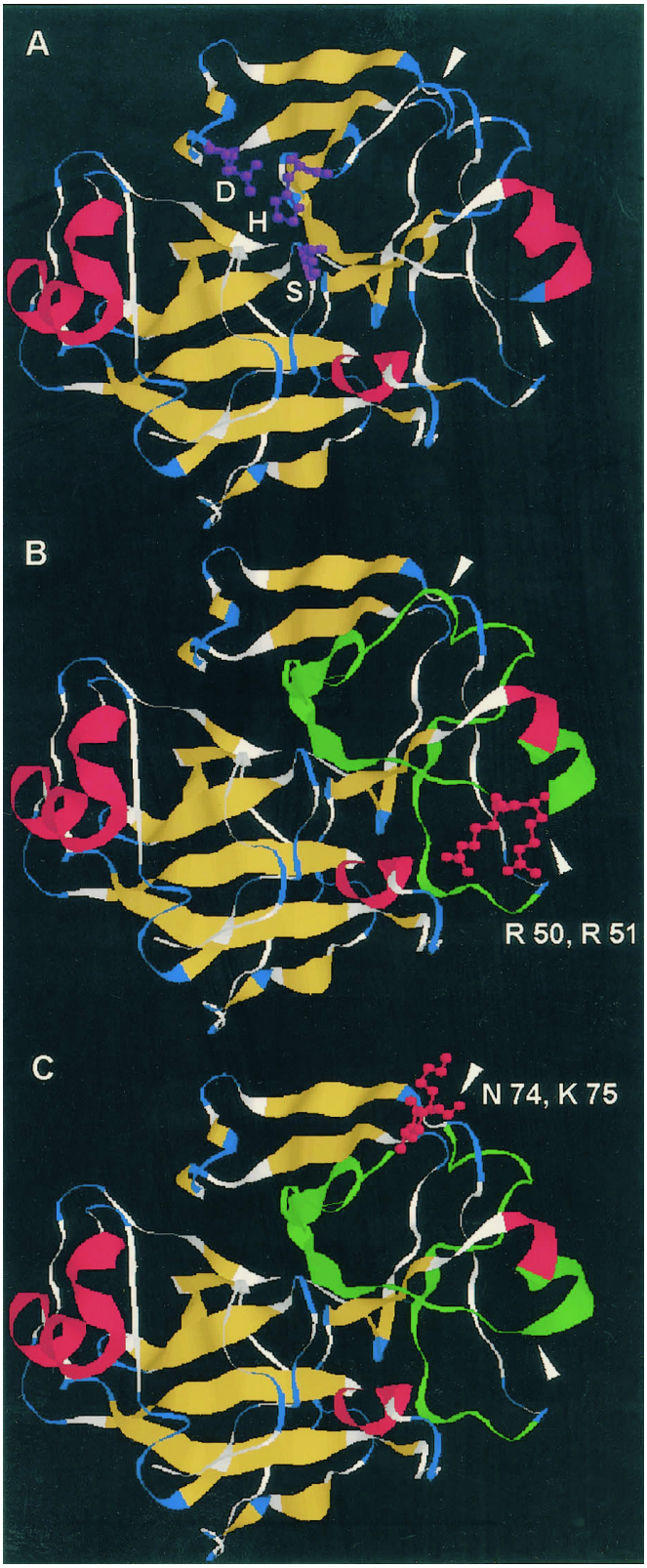


FIG. 8. **Schematic diagrams of the structure of rabbit proacrosin produced in RasMol2.** *A*, the catalytic triad is indicated by *D*, *H*, and *S*. The *arrowheads* indicate the positions on two loops containing positively charged residues that were mutated. α-helices are *magenta*, β-sheets are *yellow*, turns are *blue*, and all other residues are *white*. *B*, *arrowheads* and *colors* as in *A* except that the amino acid sequence of Ace A-2 is indicated in *green*. The two mutated resides Arg^{50} and Arg^{51} in 1 mut A are shown as *ball and stick figures*. *C*, *arrowheads* and *colors* as in *A* except that the amino acid sequence of Ace A-2 is indicated in *green*. The two mutated resides Asn^{74} and Lys^{75} in 2 mut A are shown as *ball and stick figures*.

Chymotrypsinogen A was chosen as a reference structure to do the three-dimensional protein modeling of proacrosin because its sequence contains eight Cys residues, six of which correctly align with six Cys residues of proacrosin. This results in a three-dimensional model with three Cys–Cys disulfide bonds, which correctly fold the proacrosin molecule to give the catalytic triad containing residues His^{61}, Asp^{115}, and Ser^{213} (Fig. 8). Although this model of proacrosin may not be exactly correct in all its atomic details, it allowed us to visualize the Ace A-2 sequence (Fig. 8, *B* and *C*) and its two loops, each containing either exposed arginine or lysine residues. As shown in Fig. 7*B*, mutation 1 mut A, which changed arginines 50 and 51 to alanines, no longer possessed significant zona binding activity, leading to our conclusion that these residues are within the site critical for zona binding. In contrast, mutation 2 mut A in the other loop did not affect zona binding. These results suggest that proacrosin-zona pellucida binding is primarily an electrostatic interaction, involving the negatively charged groups of ZP and positively charged residues of proacrosin. Our modeling study (Fig. 8) also indicated that the arginine residues present in the binding site would be positioned along a groove a short distance from the active site. Consequently, the sperm cell surface proacrosin/acrosin could bind zona pellucida after the acrosome reaction and then release it and the spermatozoon by subsequent proteolysis. Such a hypothesis fits into our proposed cyclic model of zona penetration (26). Indeed, recent evidence additionally suggests that as the spermatozoon penetrates, the zona concentration in its microenvironment may actually regulate proacrosin's enzymatic activity (15).

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