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ISOLATION OF THE OUTER ACROSOMAL MEMBRANE FROM BULL SPERM

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SUMMARY

A procedure is described for subcellular fractionation of bull sperm which allows the isolation of outer acrosomal membrane without the use of detergent. After washing to remove seminal plasma contaminants, the acrosomal membrane is removed by homogenization and separated on a two-step sucrose gradient. The isolated membranes have been characterized by light and electron microscopy and enzyme analysis. While the acrosomal enzymes hyaluronidase and acrosin are bound to the isolated membranes, they represent only a small percentage of the total activity and therefore do not provide reliable marker enzymes for this fraction.

Subcellular fractionation of sperm also yields information on the solubility of acrosomal enzymes. Two types of acrosomal enzymes have been identified on the basis of their distribution in gradient fractions. Both α-fucosidase and β-*N*-acetyl glucosaminidase are concentrated in the soluble fraction of the gradient. In contrast, over 70 % of the acrosin and hyaluronidase activity remains associated with the sperm pellet. These differences in solubility of these enzymes may reflect differences in their function in fertilization.

INTRODUCTION

In a recent review, McRorie and Williams [1] have summarized research on the acrosome of mammalian sperm and on the enzymes contained within this organelle. Using a variety of extraction techniques based on detergent [2] or salt [3] treatment, investigators have identified a growing number of hydrolytic enzymes in acrosomal extracts. Studies on these enzymes, particularly acrosin and hyaluronidase, have established their role in sperm penetration during fertilization [1]. Further, the acrosome reaction, which involves vesiculation of the acrosomal and plasma membranes, implicates the acrosomal membrane in the fertilization process.

In contrast to acrosomal enzymes, little work has been reported on the biochemistry of acrosomal membranes. Bernstein and Teichman [4] have correlated ultrastructural changes in the sperm with extraction of acrosomal enzymes. They reported that, after rupture of the acrosomal membrane with barbituric acid, a large fraction of the proteinase and acid phosphatase activities remained with the sperm

pellet, and that these enzymes could be solubilized by detergent. Srivastava et al. [5] found that neuraminidase is not removed from sperm by extraction with Hyamine 2389, a procedure which removes the outer acrosomal membrane. Subsequent treatment of the sperm with Triton X-100 solubilized the sperm neuraminidase, which they concluded is bound to the inner acrosomal membrane. More recently, Srivastava [3] and Srivastava et al. [6] used a sequential extraction procedure which involved incubating and homogenizing sperm in a $MgCl_2$ solution, followed by incubation in detergent. Most of the acrosin and hyaluronidase activities were found in the salt extract, which removes the outer acrosomal membrane. Lesser amounts of these enzymes were present in the detergent extract. These studies suggest that acrosomal enzymes may have different locations within the acrosome. However, as McRorie and Williams [1] have pointed out, the location of these enzymes is as yet inconclusive.

A procedure for fractionating sperm membranes, following fragmentation of the sperm by shaking with glass beads, has been described by Morton and Lardy [7] and Morton [8]. An acrosome band was separated by gradient centrifugation, however, its properties were not studied. Morré et al. [9] have isolated acrosome-rich fractions from boar sperm using detergents. They reported that material derived from the acrosomal matrix is associated with the isolated membranes. Lunstra et al. [10] have developed a procedure for purification of plasma membrane from the head of boar sperm. Finally, Multamaki [11] described a method for purifying acrosomes from bull sperm following treatment with detergent. The acrosome fraction contained acrosin and acid phosphatase activity, but not hyaluronidase. Further, acrosin was removed from the acrosomes by solubilization with Triton X-100. These results suggest either that the acrosomal enzymes are associated with the membrane or that they are trapped within the membranes. A major drawback to the use of detergent is that it can solubilize both proteins and lipids from membranes and thus affect the properties of the isolated acrosomes and complicate studies on the relationship between acrosomal enzymes and membranes.

Further research is needed to establish the extent and nature of enzyme binding to the acrosomal membrane and the location of enzymes within the acrosome. However, before such studies are undertaken, procedures are needed for isolating acrosomal membranes which do not rely on detergent for removing the sperm acrosome. This paper describes a method for purifying the outer acrosomal membrane from bull sperm without the use of detergent, and presents results on the distribution of acrosomal enzymes in subcellular fractions.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin, fraction V, α-*N*-benzoyl-L-arginine ethyl ester, hyaluronic acid, grade I, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl α-L-fucoside, *p*-nitrophenyl α-D-mannoside and *p*-nitrophenylphosphate were purchased from Sigma Chemical Co. Sucrose, analytical reagent grade, was purchased from Mallinckrodt. Other chemicals used were of reagent grade.

Isolation of sperm

Semen was collected from five Holstein bulls using an artificial vagina. For a typical acrosome preparation, approx. 30 ml of semen, collected from two bulls, were

diluted with 2 vols of Krebs-Ringer solution containing 2.77 mM fructose. Sperm were isolated from the diluted semen by a modification of the procedure of Garbers et al. [12] for isolating cytoplasmic droplets. 15 ml of diluted semen were layered on 20 ml of 1.3 M sucrose/0.9 % NaCl in conical centrifuge tubes and centrifuged for 30 min at full speed in an IEC model HNS clinical centrifuge at room temperature. The sperm pellets were resuspended in 108 ml of 0.15 M NaCl/5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.0, using a Pasteur pipette. A second washing step was carried out by layering 18 ml of the sperm suspension onto 20 ml of 1.3 M sucrose/0.9 % NaCl and centrifuging for 20 min at 20 000 rev./min in a Beckman SW 27 rotor at 0–5 °C.

Isolation of acrosomal membranes

The final pellets of washed sperm were resuspended in 60 ml of 0.15 M NaCl/5 mM HEPES, pH 7.0, and homogenized in a glass-teflon homogenizer using ten strokes at low speed. Approx. 65 % of the acrosomes are removed, as judged by phase contrast microscopy. Acrosomes were separated from other sperm fragments by placing 10 ml of homogenate on a discontinuous sucrose gradient consisting of 14 ml of 1.75 M sucrose/0.9 % NaCl and 14 ml of 1.3 M sucrose/0.9 % NaCl. The tubes were centrifuged for 3 h at 27 000 rev./min using a Beckman SW 27 rotor at 0–5 °C. The acrosomal membranes, which band on the 1.3 M/1.75 M sucrose interface, were collected with a pipette, diluted with an equal volume of buffered saline and pelleted by centrifugation for 30 min at 30 000 rev./min in a Beckman No. 30 rotor. The final pellet was resuspended in a small volume of buffered saline. In a typical preparation, a homogenate containing $2 \cdot 10^{10}$ sperm cells yielded 4–5 mg of acrosomal membrane protein.

Assays

Protein was determined by the method of Lowry et al. [13].

Glycosidase activity was assayed by a modification of the method described by Dewald and Touster [14]. The assays were performed in a final volume of 0.1 ml containing 3.3 mM *p*-nitrophenyl glycoside, 0.2 M buffer and up to 0.05 ml of sample. Acetate buffer, pH 5.5 and 6.0, was used for the assay of α-fucosidase and β-*N*-acetyl glucosaminidase, respectively, and cacodylate, pH 6.5, was used for the assay of α-mannosidase. The reaction was started by the addition of glycoside and the mixture was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.7 ml of 0.1 M glycine, pH 10.5, and the absorbance read at 400 nm. Enzyme and substrate blanks were run with each set of reactions and the absorbance was converted to nmol of *p*-nitrophenyl liberated by comparison with standard concentrations of *p*-nitrophenol.

Alkaline phosphatase activity was measured at 37 °C by recording the increase in absorbance at 400 nm using a Gilford recording spectrophotometer equipped with Beckman optics. The reaction mixture contained 5 mM *p*-nitrophenol and 50 mM glycine, pH 10.5, in a total volume of 1.0 ml.

Acrosin was assayed using α-*N*-benzoyl L-arginine ethyl ester as substrate [15]. The reaction mixture contained, in a final volume of 0.5 ml, 0.6 mM substrate, 0.1 M $CaCl_2$, and 40 mM HEPES, pH 8.0. The reaction was measured at 37 °C by recording the increase in absorbance at 253 nm. The absorbance change was converted to nmol

of substrate hydrolyzed using the absorbance difference of 1150 $M^{-1} \cdot cm^{-1}$ reported by Polakoski and McRorie [16].

Hyaluronidase was assayed by a modification of the method of Aronson and Davidson [17]. The reaction was carried out in a final volume of 0.1 ml containing 0.6 mg hyaluronic acid, 0.05 M acetate, pH 3.8, 0.075 M NaCl and 0.05 ml of sample. The reaction mixture was incubated for 20 min and the reaction stopped by boiling for 3 min. After neutralization with 0.03 ml of 0.1 M NaOH, product was measured by the method of Reissig et al. [18] using *N*-acetylglucosamine as a standard.

Electron microscopy

Samples for electron microscopy were prepared as described by Morré et al. [9]. The samples were fixed with 2 % glutaraldehyde for 1 h at 4 °C and postfixed with 1 % OsO_4 for 1 h at room temperature. After dehydration with acetone, the samples were embedded in Epon [19] or Epon-Araldite. Thin sections were stained with alkaline lead citrate [20] and examined in an RCA EMU-3G electron microscope.

RESULTS

Effect of washing sperm

The method of Garbers et al. [12], for preparing cytoplasmic droplets, was modified to serve as a washing procedure to separate sperm from other semen components. After two centrifugations through 1.3 M sucrose, most of the cytoplasmic droplets are removed from the sperm suspension. In addition to washing the sperm, the hyperosmotic sucrose causes a loosening of the acrosomal and plasma membranes on the sperm head, thus facilitating their removal. While acrosomal membranes are removed during resuspension of the sperm pellet with a Pasteur pipette after the first centrifugation, more extensive removal is achieved by homogenization.

Fig. 1a gives the appearance of the sperm homogenate in phase contrast microscopy. The equatorial segment is clearly visible as a dark band across the middle of the sperm heads, indicating that the acrosomes have been removed. Electron micrographs of the sperm homogenate, Fig. 1b, show that the plasma membrane covering the anterior portion of the head has been removed and the outer acrosomal membrane is detached at the junction with the equatorial segment. The inner acrosomal membrane and equatorial segment remain closely associated with the sperm head. It should also be noted that the inner acrosomal membrane is essentially free of the amorphous material contained within the acrosome. These results indicate the isolated acrosome fraction is derived from the outer acrosomal membrane of the sperm.

Isolated acrosomal membranes

The free acrosomal membranes present in the homogenate are separated from other sperm components on a discontinuous sucrose gradient as described in Methods. When the gradient fractions are examined under phase contrast microscope, the band of material at the interface between the sample and 1.3 M sucrose consists of small particles including some cytoplasmic droplets. The band at the 1.3 M/1.75 M sucrose interface is largely acrosomal membranes (Fig. 2a) with a few tail fragments. Intact sperm, sperm fragments and some acrosomes are found in the pellet. Additional acro-

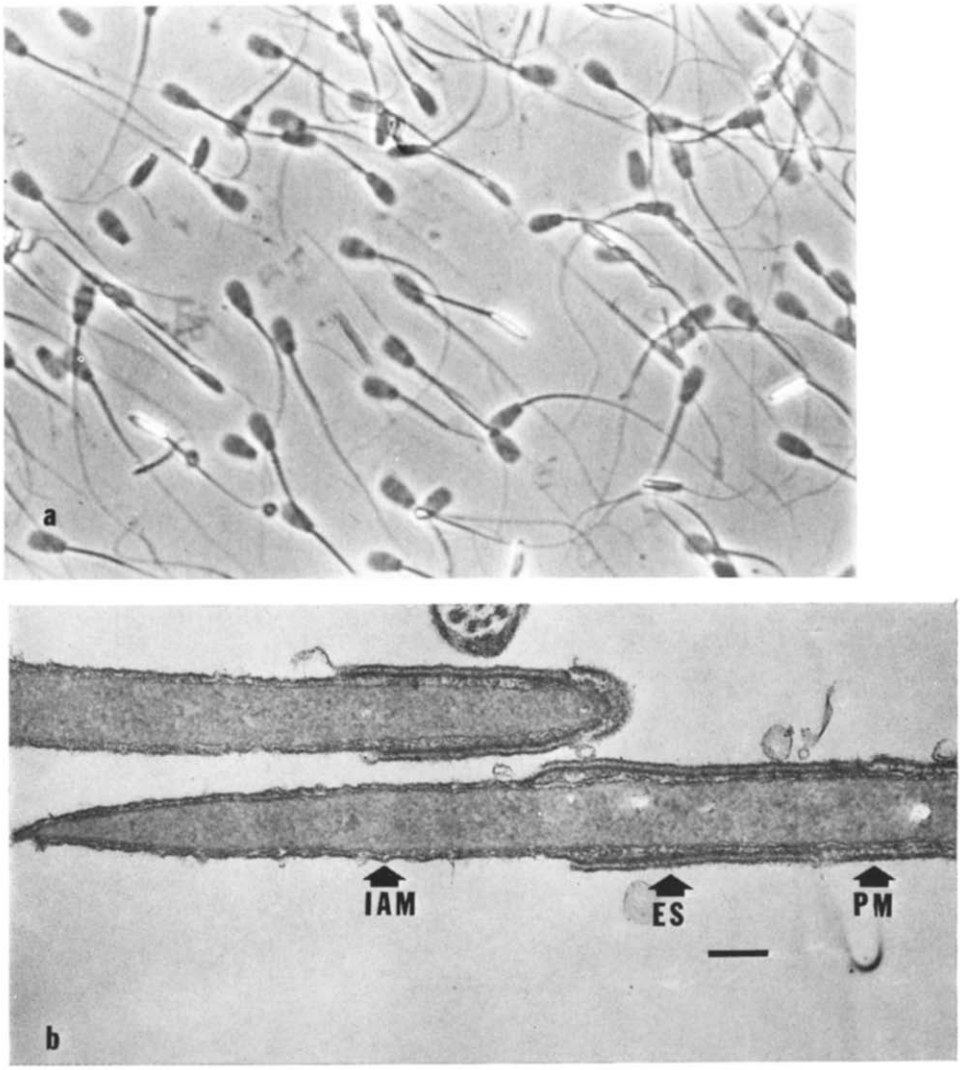

Fig. 1. Appearance of bull sperm after homogenization to remove the acrosome. (a), phase contrast micrograph of homogenized sperm, × approx. 450. (b), electron micrograph of sperm heads after homogenization. The equatorial segment (ES), inner acrosomal membrane (IAM) and plasma membrane (PM) are indicated. The bar represents 0.25 μm.

somal membranes can be isolated by recentrifugation of the sperm pellet on a second sucrose gradient.

The isolated outer acrosomal membranes retain the cap-shaped appearance typical of acrosomes, when viewed in the light microscope, Fig. 2a. In the electron micrographs, Fig. 2b, isolated acrosomes appear as membrane sheets which have amorphous material associated with their surface. This material is probably derived from the contents of the acrosome and has been demonstrated on the surface of acrosome-rich fractions obtained from boar sperm [9].

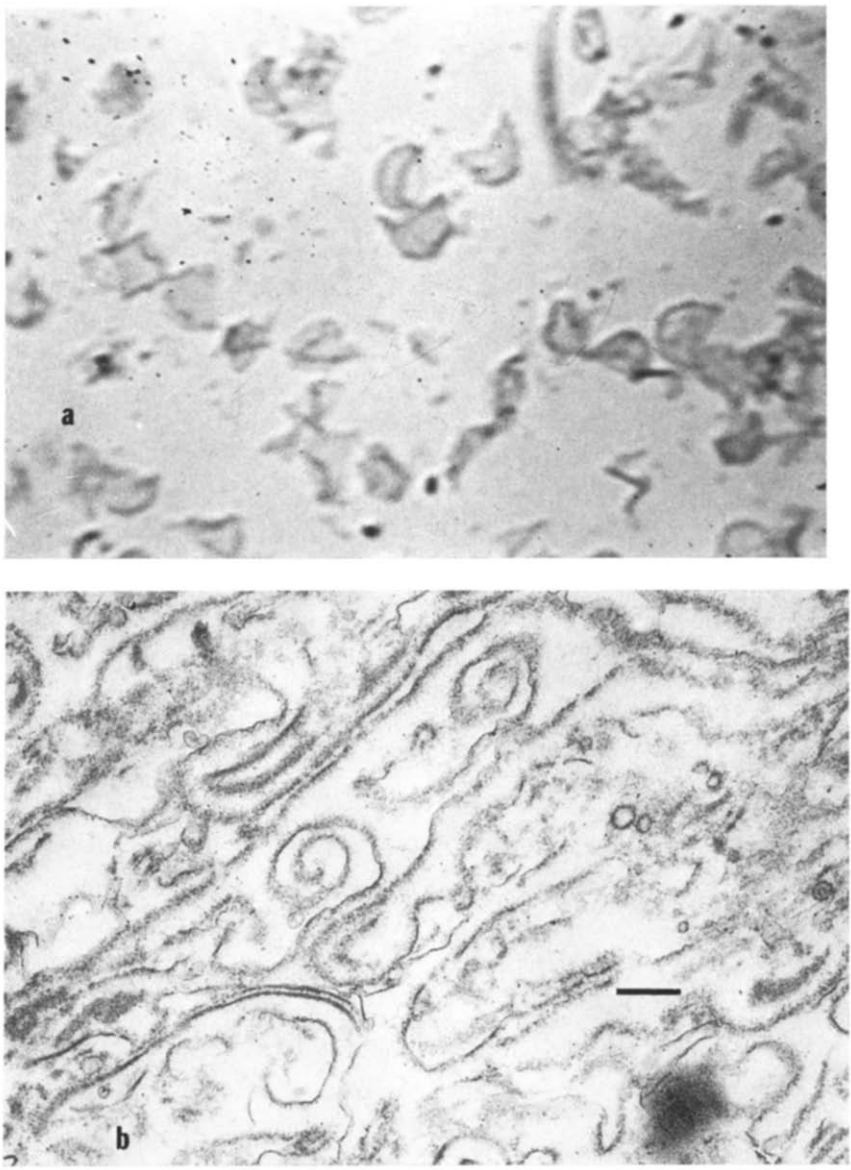

Fig. 2. Appearance of isolated outer acrosomal membranes. (a), phase contrast micrograph of the acrosome fraction, × approx. 1200. (b), electron micrograph of the acrosome fraction. The bar represents 0.25 μm.

Enzymatic activities

To aid in estimating the purity of the isolated acrosomal membranes, each gradient fraction was assayed for α-fucosidase, α-mannosidase, β-*N*-acetylglucosaminidase and alkaline phosphatase activity. Table I presents the results from a typical fractionation. The soluble fraction from the top of the gradient contains all three glucosidases activities. Of these, *N*-acetylglucosaminidase has a low specific activity in

TABLE I

ENZYMATIC ACTIVITIES IN GRADIENT FRACTIONS

Fractions were prepared from the gradient used in separating acrosomal membranes. A soluble fraction was removed from the sample volume on top of the gradient. Material at the sample 1.3 M sucrose interface (top band) and the 1.3 M/1.75 M interface (acrosome band) was pelleted and resuspended in 0.15 M NaCl/5 mM HEPES, pH 7.0. The sperm pellet was resuspended in the same sucrose solution. Specific activities are given as nmol/min per mg protein.

Fraction	α-fucosidase	α-mannosidase	β-*N*-acetyl-glucosaminidase	Alkaline phosphatase
Soluble	26.7	9.7	14.5	19.7
Top band	1.32	10.5	0.84	163
Acrosome band	3.10	0.93	0.64	10.6
Sperm pellet	2.15	0.48	1.04	1.2

the particulate fractions. The material at the sample/1.3 M sucrose interface is characterized by a high specific activity for α-mannosidase and alkaline phosphatase, enzymes which are markers for cytoplasmic droplets (Zahler, W. L., unpublished observation) and plasma membranes [21], respectively. The acrosome fraction has a relatively low specific activity for these enzymes, and with the exception of α-fucosidase, their specific activities vary considerably from preparation to preparation indicating contamination from other gradient fractions.

To determine whether acrosomal enzymes are bound to membranes, isolated acrosomal membranes were assayed for acrosin and hyaluronidase activity. Both of these enzymes are present in acrosome preparations with average specific activities of 610 and 10 nmol/min per mg protein. Further, treatment of acrosomal membranes with 1 % Triton X-100 failed to release these enzymes, suggesting they are bound to the membrane. To determine the significance of this binding, the amount of activity in the acrosome fraction was determined. Table II presents the distribution of acrosomal enzymes in the soluble, acrosome and pellet fractions. In all cases only a small percentage of the total enzyme activity is present in the acrosome fraction. Furthermore, the specific activity does not indicate an enrichment of enzymes in this fraction. The highest percentage of α-fucosidase and β-*N*-acetylglucosaminidase activity is found in the soluble fraction, as would be expected for soluble enzymes. Surprisingly, over 90 % of the acrosin and over 70 % of the hyaluronidase are in the sperm pellet. These results indicate that there are differences in the solubility and/or location of these enzymes.

DISCUSSION

This report describes a simple procedure for isolating the outer acrosomal membrane from bull sperm without the use of detergent. Removal of acrosomal membranes is accomplished by exposure of the sperm to hyperosmotic sucrose solutions during washing, which results in breakage of the outer acrosomal membrane at or near the equatorial segment. Loosening of the membranes on the sperm head is evident in phase contrast microscopy and some acrosomal membranes are removed during resuspension of the sperm after the first wash. More efficient removal is accomplished

TABLE II

DISTRIBUTION OF ACROSOMAL ENZYMES

Gradient fractions were prepared as described in Table I. Specific activity is given as nmol/min per mg protein. Total activity is given as nmol/min except for acrosin which is expressed as μmol/min. Less than 1 % of the total activity for these enzymes was found in the top band from the gradient.

Enzyme	Soluble			Acrosomal membranes			Sperm pellet		
	Specific activity	Total activity	%	Specific activity	Total activity	%	Specific activity	Total activity	%
β-*N*-Acetyl-glucosaminidase	8.5	212	66	0.71	2.4	1	0.54	105	33
α-Fucosidase	13.7	343	56	1.7	5.8	1	1.36	265	43
Acrosin	390	9.6	5	610	2	1	890	170	94
Hyaluronidase	30.1	742	26	10.3	34	1	10.6	2066	73

by homogenization of the washed sperm, which removes the acrosomal membrane from about 65% of the sperm as judged by phase contrast microscopy. After detachment of the acrosomal membrane, the equatorial segment is clearly visible in phase contrast micrographs as a dark band on the sperm head. Electron micrographs of the homogenized sperm demonstrate that the outer acrosomal membrane and the overlying plasma membrane have been removed from the sperm, while the inner acrosomal membrane remains closely associated with the sperm head, as does the plasma membrane posterior to the equatorial segment.

Identification of the isolated acrosomal membranes is based primarily on morphology. After homogenization, the detached outer acrosomal membranes retain their characteristic cap-shaped appearance in light microscopy. In addition, electron micrographs of acrosomal membranes show the presence of acrosomal material adhering to the membrane surface, as has been reported by Morre et al. [9]. This material probably accounts for the relatively high density of acrosomal membranes as indicated by sedimentation through 1.3 M sucrose. Further, the surface material may also contribute to maintenance of the morphology of isolated acrosomal membranes.

The enzymes acrosin, hyaluronidase and α-fucosidase are present in the acrosome fraction with relatively constant specific activities. Furthermore, their insolubility in Triton X-100 indicates they are firmly associated with the membrane. However, these enzymes are not reliable markers for acrosomal membranes since their activity in the acrosome fraction represents only a small percentage of the total activity and there is little if any enrichment in the acrosome fraction.

Multamaki [11] has reported the isolation of acrosomal membranes from bull sperm following detergent treatment. He showed that acrosin, but not hyaluronidase, is present in the acrosome fraction and can be solubilized by treatment with Triton X-100. When the results reported by Multamaki are compared with those reported in this paper, differences are seen in the binding of enzymes to the acrosomal membrane. These differences most likely result from the use of detergent to remove the acrosomal membrane in the procedure of Multamaki. In addition to affecting the solubility of proteins, detergents solubilize lipids and would interfere with studies of this membrane component. Thus a major advantage of the procedure described here is the absence of detergent in the isolation procedure.

The top band obtained by separation of sperm homogenates is enriched in plasma membrane, as indicated by its high alkaline phosphatase activity. Although it is contaminated by residual cytoplasmic droplets, this fraction may be useful in studies of sperm plasma membrane and as a starting material for further purification.

In addition to permitting the isolation of acrosomal membranes, the method for disrupting sperm reported here provides insight into the location and properties of acrosomal enzymes. While both hyaluronidase and acrosin are found in preparations of acrosomal membranes, the majority of the hyaluronidase and acrosin activity is found in the sperm pellet. Further, they must be associated with the sperm in order to sediment in the sucrose gradient. These results cannot be accounted for by incomplete breakage of the outer acrosomal membrane, since over half of the α-fucosidase and β-*N*-acetyl glucosaminidase activity is present in the soluble fraction. Thus it is likely that some acrosomal enzymes are present in sperm in a relatively insoluble form. Further research will be necessary to confirm this conclusion.

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