ACROSOMAL ENZYMES: IMMUNOCHEMICAL LOCALIZATION OF ACROSIN AND HYALURONIDASE IN RAM SPERMATOZOA

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Summary. Acrosin and hyaluronidase have been localized in the acrosomal region of ram spermatozoa using specific antibodies raised against the highly purified enzymes. Hyaluronidase staining was denser at the periphery of the sperm head, whereas acrosin staining was denser in the equatorial region and appeared to be bound to the inner acrosomal membrane.

It has been suggested that in animals such as the rabbit in which the cumulus cell mass is not dispersed before fertilization hyaluronidase released by spermatozoa may aid penetration through this layer (Austin, 1960). The role of hyaluronidase in animals such as sheep in which the cumulus cells are dispersed before fertilization is not known. It seems to be generally agreed, however, that acrosin (or sperm trypsin-like enzyme) is responsible for sperm penetration of the zona pellucida (Srivastava et al., 1965; Stambaugh et al., 1969; Zaneveld et al., 1973; Polakoski & McRorie, 1973), but the evidence that these particular enzymes are even found in the acrosome has been largely circumstantial. It has been based on changes in sperm morphology after enzyme extraction (Pedersen, 1972; Brown & Hartree, 1974; Churg et al., 1974; Srivastava et al., 1974), on the localization of labelled non-specific enzyme inhibitors (Stambaugh & Buckley, 1972) and on the detection of proteolytic activity using gelatin films (Gaddum & Blandau, 1970; Benitez-Bribiesca & Velazquez-Meza, 1972; Gaddum-Rosse & Blandau, 1972; Penn et al., 1972; Allen et al., 1974). This proteolytic activity is unlikely to be specific as there is increasing evidence that more than one proteinase exists in spermatozoa (Dott & Dingle, 1968; Allison & Hartree, 1970; Multimaki & Niemi, 1972; Yanagimachi & Teichman, 1972; Bernstein & Teichman, 1973; Polakoski et al., 1973; Srivastava & Foley, 1973).

Another approach to the problem lies in the use of monospecific antisera to localize a particular enzyme. This technique has the advantage that the purified antibodies are highly specific protein markers and control experiments can be carried out using antibodies from non-immune sera.

As part of a study on the function of the acrosomal enzymes, acrosin and hyaluronidase have been localized at the light microscope level using specific antibodies. These two enzymes were purified to a high degree from ram semen (acrosin: 35 i.u./mg Nα-benzoyl-DL-arginine-p-nitroanilide HCl; hyaluronidase: 375...
20 i.u./mg with the end group assay) by methods similar to those previously described (Morton, 1973), but contaminants were still present as judged by sodium dodecyl sulphate electrophoresis, isoelectric focusing on polyacrylamide gels, Ouchterlony gel diffusion plates and rocket immunoelectrophoresis. Specific antisera were raised in rabbits by cutting out rabbit antibody–sheep antigen precipitin lines from agar gels and using these as immunogens. Antisera were rigorously tested against contaminants as well as against purified enzyme for specificity (using gel diffusion and rocket immunoelectrophoresis techniques and staining the precipitin lines for protein and enzyme activity) and only apparently specific sera were subsequently used. Specific antibodies against ovine acrosin did not cross-react at pH 7·3 or pH 5·0 with bovine, ovine or porcine pancreatic trypsins but did give a line of identity with bovine acrosin (see Stambaugh & Smith, 1974).

In these experiments the Fab monomer fragment of IgG was used at all stages as it was found that even control IgG bound non-specifically to spermatozoa: the non-specific binding was abolished when the purified antibody-binding fragment of IgG was used. Freshly ejaculated, or epididymal, ram spermatozoa were diluted ×20 to ×40 with phosphate-buffered saline containing 1% formaldehyde. After 5 min, the diluted semen was smeared onto a glass slide and allowed to dry. Slides were then washed in phosphate-buffered saline and the specific antibody, or the same amount of control antibody, applied. Slides were again washed and a second antibody (specific for the species of Fab used in the first step, i.e. pig anti-rabbit Fab) applied, only this time labelled with either fluorescein isothiocyanate (Thé & Feltkamp, 1970) or horseradish peroxidase (Avrameas & Ternynck, 1971; Mannik & Downey, 1973). Staining for peroxidase activity was carried out with either 3,3-diaminobenzidine tetrahydrochloride or 3-amino-9-ethylcarbazole (Graham et al., 1965; Graham & Karnovsky, 1966).

Spermatozoa treated with antibody to either enzyme became stained over the anterior region of the head. Spermatozoa treated with anti-hyaluronidase antibody were more stained at the periphery, whereas those treated with anti-acrosin antibody were stained more densely in the equatorial region and staining extended further down the sperm head (Plate 1). Further evidence that supports this apparent difference in enzyme location was obtained when spermatozoa were treated in ways leading to a partial loss of enzyme staining, e.g. overfixation with too great a concentration of fixative or too long a period of fixation, or low concentration of antibody. In these experiments, hyaluronidase staining persisted at the periphery of the sperm head whereas acrosin staining persisted in the equatorial region.

Resolution at the light microscope level, however, does not allow precise localization of these enzymes to be made. For example, the enzyme may be in the acrosomal contents, bound to an acrosomal membrane or indeed, may even be sub-acrosomal or just beneath the plasma membrane. Evidence for a more exact localization can be obtained by removing or disrupting both the plasma membrane over the acrosome and the outer acrosomal membrane, thus releasing the acrosomal contents. This was effected by hypotonic shock followed by freezing and thawing and mixing on a vortex mixer. In these prepara-
Staining of ram spermatozoa using specific antibody and immunoperoxidase labelling (aminoethyl carbazole reaction, ×1200). Spermatozoa after treatment with: antibody to hyaluronidase (Fig. a), antibody to acrosin (Fig. b), and non-specific antibody (Fig. c).

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tions, ultrastructural examination showed that the inner acrosomal membrane and equatorial segment remained intact. It was found that about 80% of the hyaluronidase activity was eluted from the spermatozoa in the subsequent washings and this was coincident with a marked reduction in hyaluronidase staining of the sperm head. On the other hand, about 80% of the acrosin activity remained bound to the spermatozoa and only a slight decrease in immunochemical staining was observed. Solubilization of residual acrosin and hyaluronidase activities using magnesium chloride or detergent did not obviously affect the inner acrosomal membrane or equatorial segment but abolished sperm staining with either antibody almost completely.

The present results suggest that hyaluronidase is mainly present within the acrosomal contents whereas acrosin (and possibly some hyaluronidase) is mainly bound to the inner acrosomal membrane. Recently, Gould & Bernstein (1973, 1975) localized hyaluronidase with immunochemical methods at the electron microscope level and their results also suggest that hyaluronidase is located mainly in the acrosomal contents. Selective membrane removal followed by ultrastructural localization studies may confirm that acrosin and hyaluronidase are bound to the inner acrosomal membrane.

The demonstration of enzymes bound to the inner acrosomal membrane of spermatozoa might explain how spermatozoa that have undergone the acrosome reaction are able to penetrate between the cumulus cells (as in the mouse and rabbit) and through the zona pellucida. The potential use of specific antibodies as enzyme inhibitors may also clarify the roles of these acrosomal enzymes.

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