gram in figure 1, f, was constructed from the data in figure 1, a–e, and shows the proportion of unmyelinated axons in the nerve which fall into each 0.15 μm histogram class. Thus almost three-quarters (73%) of unmyelinated axons have diameters from 0.3 to 0.6 μm. There is a possibility that the preparative procedures used in this study may have changed the size and shape of the axons. Extrapolations from studies on mammalian myelinated fibers suggest that the axons may have either shrunk radially by about 8% or increased in diameter by, on average, 17.3%. However the validity of such extrapolations is somewhat questionable.

b) *Conduction velocity*. As the intensity of the stimulus applied to the nerve trunk was raised, a small deflection was recorded which increased in amplitude to a maximum. In all five preparations a large diphasic wave-form such as that illustrated in figure 2, f, constituted the largest component of the compound action potential. This component also had the lowest threshold (fig. 2, a–e). The peak conduction velocity of this component ranged from 0.5 (fig. 2) to 0.68 $m \cdot s^{-1}$ (mean 0.62 ± 0.031 (SEM) $m \cdot s^{-1}$), which suggests that it is the result of excitation of C-fibers. Since it was also the largest component in the compound action potential, this also suggests, that unmyelinated fibers predominate within the nerve trunk. This agrees with the ultrastructural findings. Other smaller components with conduction velocities either slower or faster than that of the main component were also seen in some preparations.

The relationship between unmyelinated nerve fiber diameter (D) and conduction velocity (CV) had been examined previously in the squid (giant axon $CV = D^{0.57}$ at 20 °C)[7]; the cockroach ($CV = D^{0.78}$ at 20 °C)[8] and the cat ($CV = D$ at 37 °C)[9]. A mean unmyelinated nerve fiber diameter of 0.502 μm and a mean peak conduction velocity of 0.62 $m \cdot s^{-1}$ produces a relationship of $CV = D^{0.69}$ for chicken unmyelinated fibers. This result supports the hypothesis that the relationship between CV and D is a higher power than expected from purely dimensional considerations[10].

Since there have been very few analyses of the composition and size distribution of nerve fibers in avian autonomic nerves[11] the results of the present study should, therefore, be of interest to avian physiologists and anatomists especially those investigating the intestine and its innervation.

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0014-4754/85/070919-03$1.50 + 0.20/0

Localization of vitelline-coat lysin purified from testis of a top shell, *Turbo cornutus*

A. Ogawa and K. Haino-Fukushima[1]

Department of Biology, Faculty of Science, Tokyo Metropolitan University, Fukasawa, Setagaya-ku, Tokyo 158 (Japan), 2 July 1984

Summary. The vitelline-coat lysin purified from the testis of *Turbo cornutus* was found, by an immunofluorescence technique, to be located in the acrosome of the sperm, which suggested that the lysin reacts with the vitelline-coat in an early phase of fertilization to allow the sperm to penetrate through the coat.

Key words. Top shell, *Turbo cornutus;* lysin, vitelline-coat; testis; fertilization; sperm acrosome.

Sperm acrosin (EC 3.4.21.10), an acrosomal trypsin-like enzyme, is essential for sperm penetration through the zona pellucida of the ovum of mammals during fertilization[2]. In some marine invertebrates, including sea urchins[3] and acidians[4,5], hydroxylases such as chymotrypsin-like and trypsin-like proteinases have been reported to participate in the penetration of sperm through the egg investments. Also, a protein that reacts with the vitelline-coat in a stoichiometric, nonenzymatic manner has been purified from the sperm of an abalone, *Haliotis rufescens*[6], and from the testis of sea snails, *Tegula pfeifferi*[7] and *Turbo cornutus*[8]; this protein (vitelline-coat lysin) has been thought to play a role in the penetration of sperm through the coat. These vitelline-coat lysins seem to be located in the acrosomes and to be released from the vesicles accompanying acrosome reaction. But direct evidence has been found so far only in *Haliotis rufescens*[6].

We previously reported the purification of a vitelline-coat lysin from the testis of *T. cornutus*, and its properties[8]. In the present study, we prepared the antiserum to the purified lysin and located the lysin in the acrosome of the sperm by an immunofluorescence technique.

Materials and methods. The vitelline-coat lysin was purified from the testis of *T. cornutus* as described previously[8], and the final preparation appeared to be homogeneous on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Dulbecco's phosphate-buffered saline without Ca^{++} or Mg^{++} (PBS) was purchased from Nissui Seiyaku Co. and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Wakoi Pure Chemical Industries. Goat antiserum to rabbit γ-globulin (Seikagaku Kogyo Co.) was labeled with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory) by the method of Kawamura[9]. A rabbit was bled from the ear vein to obtain normal serum, immunized with purified lysin (1.4 mg each of lysin was injected four times at intervals of 10 days with Freund's complete adjuvant, Difco) and then bled to obtain antiserum. A γ-globulin fraction (antilysin antibody) was purified from the antiserum by the method of Palacios et al.[10]. The equivalence point of the antibody to the lysin was determined by the method described by Garvey et al.[11]. To obtain the antilysin antibody which had been absorbed by the lysin, antilysin antibody (1 mg of γ-globulin/50 μl in PBS) was mixed with an excess of purified lysin (10 μg/15 μl in 0.03 M potassium phosphate-buffer con-

Immunofluorescence staining of the sperm of *Turbo cornutus*. Top, fluorescence micrographs; bottom, phase contrast micrographs of the same sperms. Sperm was incubated with antilysin antibody (a), antilysin antibody that had been absorbed by excess amount of lysin (b), or normal rabbit serum (c), and then stained with FITC-labeled goat antirabbit γ-globulin antibody. Bar 2 µm.

taining 0.5 M KCl, pH 8.0), left overnight at 4 °C and centrifuged at 5000 × g for 15 min; the supernatant (lysin-absorbed antilysin antibody) was used.

Sperms from the resected testis of *T. cornutus* were suspended in 200 ml of artificial seawater and washed twice with the seawater by centrifugation at 3000 × g for 10 min. The sperm were placed on glass slides, fixed in a fixation mixture (25 mM sodium phosphate buffer, pH 7.0, containing 0.35% glutaraldehyde, 1.4% Tris, 1% EDC and 0.4 M KCl) for 7 min at room temperature by a modification of the method of Willingham and Yamada[12], and washed with PBS. After treatment with PBS containing 0.2% Triton X-100 and 300 mM glycine for 30 min at room temperature, the samples were incubated with antilysin antibody (1 mg of γ-globulin/ml in PBS containing 0.2% triton X-100 and 300 mM glysine) overnight at 4 °C. Some samples were incubated with the lysin-absorbed antibody (1 mg/ml) or normal serum (diluted to 10%), instead of antilysin antibody. Next the samples were washed with PBS, incubated with FITC-labeled goat antibody to rabbit γ-globulin (1 mg/ml in PBS) for 1 h at room temperature, washed with PBS, and mounted under 50% glycerol in 50% PBS with cover slips. They were observed with a fluorescence microscope.

Results and discussion. Antibody against the lysin purified from the testis was prepared in a rabbit. The antilysin antibody formed a single precipitation line with a crude extract of the testis and purified lysin in the Ouchterlony test. Immunoprecipitate was obtained by mixing antilysin antibody and partially purified lysin (hydroxyapatite fraction I, see Ogawa and Haino-Fukushima[8]), and subjected to SDS polyacrylamide gel electrophoresis according to the method of Laemmli[13]. Three main protein bands, originating from the lysin, light- and heavy-chains of γ-globulin, were detected on the gel. These results indicated that the antibody was specific to the lysin. The equivalence point of the antilysin antibody to purified lysin was estimated to be about 6 µg of lysin per 1 mg of the globulin.

The localization of the lysin in the sperm was examined using the antilysin antibody. Sperms of *T. cornutus* were stained by an indirect immunofluorescence technique. As shown in the figure (a), both the acrosome and the mid-piece of the sperm were stained with the antilysin antibody and FITC-labeled goat antirabbit γ-globulin antibody. The sperm indicated with the buffer without antilysin antibody previously absorbed by the lysin (b) or with the normal serum (c) showed fluorescence in the mid-piece. These results suggest that the lysin is localized in the acrosome of the sperm, and that the fluorescence in the mid-piece is caused by nonspecific adsorption of the rabbit γ-globulin to the sperm.

We previously purified a vitelline-coat lysin from the testis of *Turbo cornutus* and reported that it was not a proteinase but a protein which reacted with the vitelline coat by a stoichiometric, nonenzymatic mechanism, like *Haliotis*[6] and *Tegula*[7] lysins[8]. However, the action of the *Turbo* lysin was milder than those of the other two lysins. *Haliotis* lysin completely dissolved the vitelline coat and *Tegula* lysin solubilized a part of it, while *Turbo* lysin only loosened the fibrous structure of the vitelline coat without releasing any soluble product from it. Because of the mildness of the action, the question arose of whether *Turbo* lysin plays a role in penetrating the coat. In this study, we found that *Turbo* lysin is located in the acrosome of the sperm, which suggests that it does play a role in sperm penetration during the early phase of fertilization. In abalone, the substance in the acrosome is released though an acrosome reaction and dissolves the vitelline coat[14]. The *Turbo* lysin may be released similarly and react with the vitelline coat.

Whole acrosomes were stained by immunofluorescence staining. An acrosome contains two distinct components, an electron-dense and a less dense material, according to electron microscopic observation of *Turbo* sperm[15], like *Haliotis* sperm[16]. To clarify the relationship of the lysin and the other component, the localization of the lysin should be further investigated by electron microscopy.

1 We are grateful to Professor M. Akino (Tokyo Metropolitan University) for his helpful advice.

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