BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **234,** 54–58 (1997)
ARTICLE NO. RC976576

A Nonglycosylated, 68-kDa α-L-Fucosidase Is Bound to the Mollusc Bivalve *Unio elongatulus* Sperm Plasma Membrane and Differs from a Glycosylated 56-kDa Form Present in the Seminal Fluid

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Received March 28, 1997

The male reproductive system of the mollusc bivalve *Unio elongatulus* contains two distinct forms of α-L-fucosidase, one present in the gonad fluid and a second one associated with the sperm plasma membrane. Both activities were purified to homogeneity. The soluble seminal plasma enzyme had an oligomeric MW of 56 kDa as determined by MALDI-TOF mass spectrometry, whereas the enzyme purified from sperm plasma membranes had an MW of 68 kDa. Analyzed by lectin blotting with ConA and PNA, the 68 kDa enzyme did not bind either lectin, whereas the 56 kDa form bound ConA only. Both fucosidases followed a Michaelis-Menten kinetics with the K_m of the sperm-bound enzyme being 7.1×10^{-4} M and that of the seminal enzyme being 9.1×10^{-4} M. Both had a pH optimum of 5.0.

α-L-fucosidase is an ubiquitous glycosidase that has been studied in a variety of organisms including bacteria. Its ubiquity is due to the natural occurrence of a diverse and widespread group of fucose-containing glycoconjugates from which fucose can be hydrolyzed by the enzyme. In human pathology, the importance of fucosidase is mainly associated with the neurovisceral storage disease, fucosidosis (1,2). In recent years this enzyme has been found on the sperm membrane of tunicates (3,4) and mammals (5). This implies that it is involved in sperm-egg interaction, the process which assures species-specificity in fertilization and triggers subsequent steps leading to successful fertilization. There is also evidence that other glycosidases such as β-N-acetylglucosaminidase (6-9) and α-D-mannosidase (10) play a similar role in fertilization. In all cases, the glycosidase on the sperm has been found to match the functional sugar residues of the egg coat glycoproteins. In a previous study, we showed that the ligand for sperm binding in the mollusc bivalve *Unio elongatulus* is a 220 kDa glycoprotein and that fucose residues of its oligosaccharide chains play a key role in the interaction mechanism (11,12). We postulated that α-L-fucosidase could be a receptor molecule on the sperm. In this report, we demonstrate that two forms of α-L-fucosidase, one soluble and the other bound to the sperm membrane, are present in the gonads of *Unio elongatulus*. The two forms were purified and characterized and their oligomeric mass established by SDS-PAGE and MALDI TOF-MS. The presence of glycan chains was tested by lectin-blotting using the lectins from *Canavalia ensiformis* (ConA) and *Arachis hypogaea* (PNA).

Abbreviations: MALDI-TOF-MS: Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry; PMSF: phenylmethylsulfonyl fluoride, SF, MOPS 3-(N-morpholino)propanesulfonic acid; PBEP: sodium phosphate buffer containing 1mM EDTA and 1mM PMSF.

MATERIALS AND METHODS

Chemicals. p-Nitrophenyl-α-L-fucopyranoside, L-fucosylamine-derivatized 4% agarose for affinity chromatography and biotin-labeled lectins were from Sigma (St. Louis, MO). Digoxigenin (DIG)-labeled lectins, anti-DIG conjugated antibodies, 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride were from Boehringer-Mannheim, Germany. All other chemicals were reagent grade.

Sperm and gonad fluid sampling. Specimens of *Unio elongatulus* were collected near Siena (Italy). The gonads of live animals were teased apart in Petri dishes and the sperm collected by aspiration with a Pasteur pipette. After filtration on cheesecloth to remove tissue debris, the sperm were diluted 1:20 with filtered freshwater (FFW) containing 1 mM PMSF and 1 mM EDTA and washed by repeated centrifugation at 2000 rpm for 10 min until free of any residual gonad fucosidase activity. The sperm pellet was used as starting material for the purification of the sperm-associated α-L-

0006-291X/97 $25.00

fucosidase. The gonad fluid was obtained from the cheesecloth filtered sperm by low g centrifugation.

Preparation of spermatozoan plasma membrane vesicles. Plasma membrane vesicles were prepared from washed *Unio* sperm essentially as described by Rufo et al. (13). 1 ml aliquots of pelleted sperm were resuspended in 5 ml of 0.25 M sucrose, 0.2 mM $MgCl_2$, 10 mM MOPS, pH 7.4, placed in a Parr disruption cell (Parr Instrument Co. IL) and subjected to a nitrogen pressure of 750 psi for 15 min. After extrusion, the suspension was washed twice at 600 × g for 10 min; the combined supernatants were then centrifuged at 6000 × g for 10 min. The supernatant fraction thus obtained was centrifuged at 100,000 × g for 1 h and utilized for further purification. Part of the pellet was fixed in 2.5% glutaraldehyde, embedded in SPURR resin and checked for purity by electron microscope observation. Sperm and sperm plasma membrane preparations were carried out at 4 °C.

Enzyme assay. The activity of the enzyme was assayed by measuring the quantity of nitrophenol liberated from p-nitrophenyl-α-L-fucoside. The enzyme was incubated at 40 °C for 1 h with McIlvaine buffer, pH 5.0, containing 3 mM p-nitrophenyl α-L-fucopyranoside in a total volume of 400 μl; 1 ml of 1 M sodium carbonate was added to stop the reaction. The release of p-nitrophenol was determined by measuring the absorbance at 401 nm; 1 unit of enzyme activity was defined as the amount which liberated 1 μmole of p-nitrophenol/min. Specific activity was expressed as units/mg of protein. Protein was determined by the method of Bradford (14) with bovine serum albumin as the standard. For kinetic studies, enzyme aliquots were incubated in duplicate for 15 min at 40 °C in 0.2 ml of 0.1 M McIlvaine buffer, pH 5.0, containing p-nitrophenyl-α-L-fucoside at concentrations ranging from 0.5 to 4 mM. For the pH dependence studies, aliquots of purified enzyme preparations and p-nitrophenyl-α-L-fucoside as substrate were incubated at 40 °C for 30 min at pH values between 3.0 and 8.0 using 0.1 M McIlvaine buffer. Contaminant glycosidases were determined using the corresponding p-nitrophenyl derivatives of the sugar under their optimal conditions.

MALDI TOF-MS. The purified α-L-fucosidases were lyophilized and resuspended in distiled water to reach a concentration of 1 mg/ml. For MALDI (Matrix Assisted Laser Desorption/Ionization) analysis, sinapinic acid was used as matrix (100 mM in methanol/water, 50:50 v/v). For all experiments 5 μl of matrix was mixed with the same volume of the protein solution at a concentration of about 10 pmol/μl. About 1 μl of the resulting mixture was deposited on a gold-plated sample holder and dried under vacuum before analysis in the mass spectrometer. External calibration was performed with Hewlett-Packard G2053A Protein Standard solution and checked daily. MALDI measurements were performed in positive linear mode with a Hewlett-Packard G2025A MALDI-TOF system (Palo Alto, CA), equipped with a LeCroy 9350AM oscilloscope. Ions formed by a pulsed UV laser (nitrogen laser, λ=337 nm) were accelerated at 28 keV.

SDS-PAGE and lectin blotting. Purified α-L-fucosidases were electrophoresed in 12% polyacrylamide slab gels according to Laemmli (15). For lectin binding studies purified enzyme preparations were electrophoresed as described above and then transferred to a nitrocellulose membrane according to the method of Towbin et al. (16). The nitrocellulose was blocked overnight in TBS (Tris-HCl 50 mM, pH 7.5, NaCl 0.15 M) containing 2% bovine serum albumin and then washed three times with TBS. For the specific detection of glycan moieties, the sheets were treated with digoxigenin (DIG)-labeled lectins (17) or with biotin-labeled lectins for 1 h at the following concentrations: ConA, 0.008 μg/ml; PNA, 1 μg/ml. After three washings in TBS the sheets were incubated for 1 h in anti-DIG antibody conjugated with alkaline phosphatase at an enzyme concentration of 1U/ml or with avidin conjugated with peroxidase at a concentration of 4 μg/ml. After three washings the nitrocellulose was incubated in a stain solution consisting of 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride to develop alkaline phosphatase activity or 0.3% 4-chloro-1-naphthol in methanol and 0.01% H_2O_2 for peroxidase activity. The reactions were stopped by washing in distilled water after about 5 min. Controls were carried out by omitting the lectin incubation step or by incubating the sheets with the lectin in the presence of 0.2 M competing haptenic sugar.

RESULTS AND DISCUSSION

Release of α-L-Fucosidase from Sperm

Intact sperm prepared as described in Methods were shaken gently at 4 °C in either 0.7 M KCl in FFW or 10 mM Tris-HCl, pH 7.3, 1 mM PMSF, 1 mM EDTA, 5 mM β-mercaptoethanol and 0.1% Triton X-100. After centrifugation at 105,000 × g for 20 min, the resultant supernatant fluids and pellets were assayed for α-L-fucosidase. In the absence of Triton X-100 and in the presence of all other components of the extraction buffer, no recoverable activity was found, whereas 90% activity was recovered with the Triton-containing extraction buffer. When sperm plasma membranes were prepared, high speed centrifugation of sperm following nitrogen cavitation yielded a pellet containing 50% of the fucosidase activity recovered.[1]

Enzyme Purification

Washed spermatozoa and purified sperm plasma membranes were resuspended in solubilization buffer (10 mM Tris-HCl, pH 7.3, 1 mM PMSF, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1% Triton X-100) at 4 °C for 2 h and then centrifuged at 105,000 × g for 20 min. The pellet was re-extracted for 30 min and solid $(NH_4)_2SO_4$ was added to 60% saturation to the combined supernatants and left stirring overnight at 4 °C. After centrifugation at 105,000 × g for 40 min, the precipitate was dissolved in 500 μl of 0.1 M PBEP, pH 6.8, and dialysed extensively against the same buffer. The seminal fluid obtained as described was ammonium sulfate fractionated under identical conditions. The enzyme preparations were then applied to a Sephadex G-200 column (1,5×90 cm) equilibrated and eluted with 0.1 M PBEP, pH 6.8. Fractions (1.4 ml) were collected at a flow rate of 9 ml/h and assayed for fucosidase activity. Tubes containing enzyme activity were pooled, brought to 80% saturation by adding solid $(NH_4)_2SO_4$ and left stirring overnight at 4 °C. After centrifugation at 105,000 × g for 40 min, the precipitate was dissolved in 10 mM PBEP, pH 5.5, and dialysed extensively against the same buffer. Final purifi-

[1] 45% of the remaining activity was associated with a fluffy layer which was present at the top of the tube after the first centrifugation. The activity could be recovered in the pellet upon successive centrifugations of the supernatant. The fucosidase preparation used in the present study was derived from the pellet originating from the first centrifugation only. It consisted almost exclusively (90%) of plasma membranes, as judged by electron microscopy.

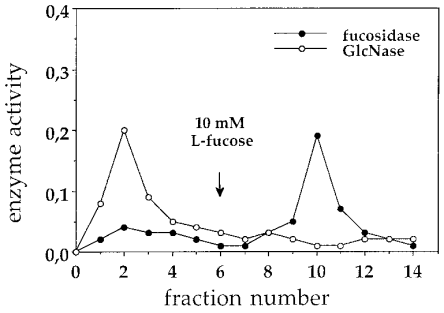

FIG. 1. Affinity chromatography of *Unio* sperm fucosidase. The arrow indicates the fraction at which α-L-fucose was added.

cation was achieved by affinity chromatography using a column (1 × 10 cm) of fucose-derivatized agarose previously equilibrated with 10 mM PBEP, pH 5.5. The dialysed samples (volumes always less than 0.5 ml) were loaded onto the column and the flow stopped for 30 min. After washing with 10 column volumes of the equilibrating buffer, bound enzyme was eluted with 10 mM α-L-fucose in the same buffer; fractions were dialyzed against the equilibration buffer to eliminate fucose and assayed for enzyme activity. The active fractions were pooled, brought to 80% saturation by adding solid $(NH_4)_2SO_4$ and left stirring overnight at 4 °C. After centrifugation at 105,000 g for 40 min, the precipitate was dissolved in 10 mM PBEP, pH 5.5, and dialysed extensively against the same buffer. The purified enzymes could be stored at 4 °C for at least one month.

The *Unio* reproductive system was found to contain various glycosidases detectable in washed spermatozoa and seminal plasma. The principal activity was β-N-acetylglucosaminidase, followed by α-fucosidase and, to a minor extent, α-mannosidase, and α- and β-galactosidase activities. The last purification step completely separated β-N-acetylglucosaminidase and α-L-fucosidase activities (Fig.1).

Characterization of U.elongatulus Fucosidases

The fucosidases from washed spermatozoa and purified sperm plasma membranes gave a single SDS PAGE band with an M_r of 69 kDa. No other contaminating band was observed in either case. Fucosidase from gonad fluid also gave a single band with an M_r of 55 kDa (Fig.2). Matrix-Assisted Laser Desorption Mass Spectrometry analysis confirmed the presence of a single polypeptide, producing a single set of molecular ions, for all the fucosidases tested (Fig.3). The average of the $(M+2H)^{2+}$, $(M+H)^+$ and $(2M+H)^+$ peaks gave an M_r of 67,847 for the sperm-bound enzyme and an M_r of 55,971 for the gonad enzyme. When analyzed by lectin blotting with ConA (specific for the core portion of N-linked oligosaccharide chains (18, 19)) and PNA (specific for O-linked chains (20)) the 68 kDa enzyme did not bind either lectin, whereas the 56 kDa form bound ConA only (Fig.2, panel B). The dependence of enzyme activity on substrate concentration followed Michaelis-Menten kinetics for both fucosidases, the K_m of the sperm-bound enzyme towards the artificial substrate being 7.1×10^{-4} M, and that of the gonad enzyme being 9.1×10^{-4} M (Fig.4). The dependence of both enzyme activities on pH was also investigated. Although the enzymes exhibited a similar pattern, with an optimum at pH 5.0, the gonad fucosidase had a sharper pH dependence curve (data not shown). The enzyme activity also varied with temperature at pH 5.0, with maximum activity was reached at 50-60 °C (data not shown).

When we originally quantified the fucosidase activity on sperm of *Unio elongatulus*, we found that α-L-fucosidase activity was one of the highest glycosidase activities associated with intact sperm, together with β-N-acetylglucosaminidase, and that the seminal plasma also contained fucosidase activity. The fact that approximately 90% of the activity was extracted with the solubilization buffer indicates that fucosidase is tightly associated with the sperm plasma membrane. Nitrogen cavitation experiments showed that 50% of the α-L-fucosidase is associated with the sperm pellet fraction. The purification procedure of sperm, sperm plasma membranes and seminal fluid all gave homogeneous preparations of the enzyme. The last affinity chromatography step removed all contaminants, including the high β-N-acetylglucosaminidase activity. When analyzed by SDS-PAGE, MALDI-MS and lectin blotting, the fucosidases purified from intact sperm and sperm

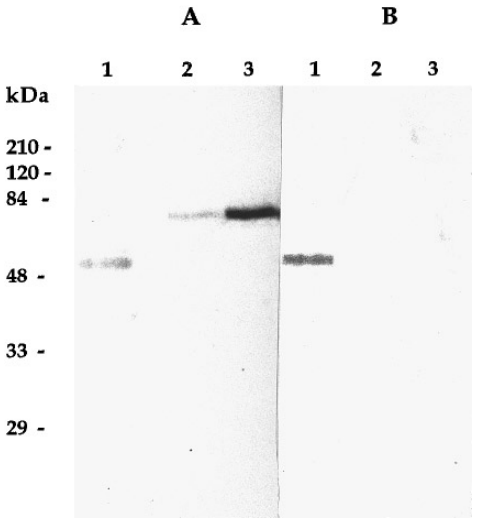

FIG. 2. SDS-PAGE and lectin blotting of purified *Unio elongatulus* sperm and gonad α-fucosidases. The electrophoresed proteins of panel A have been blotted onto nitrocellulose and probed with DIG-ConA (panel B). Panel A and B: lane 1: fucosidase from seminal plasma; lane 2: fucosidase extracted from spermatozoa; lane 3: fucosidase from plasma membrane vesicles.

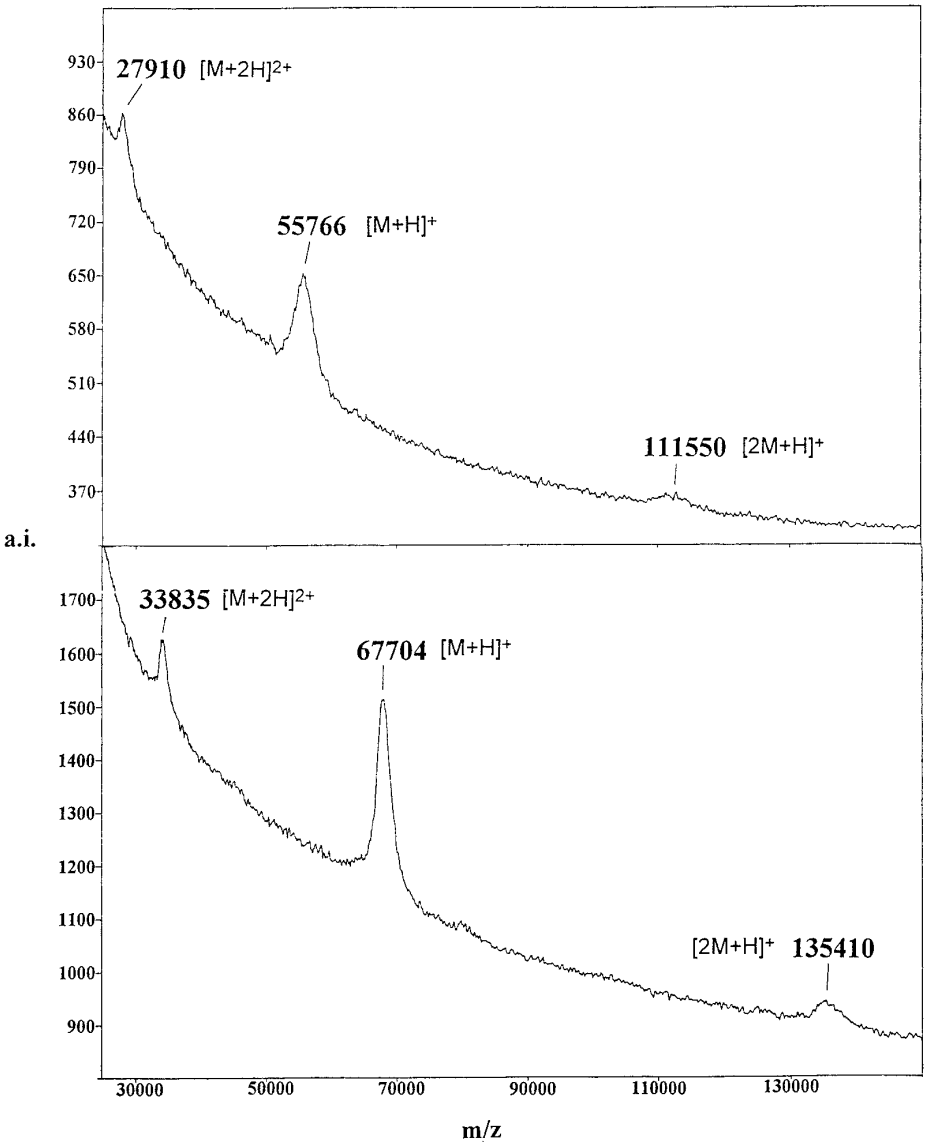

FIG. 3. Determination of the molecular mass of the two α-fucosidases from *Unio elongatulus* reproductive system. Panel A: mass spectrometry pattern for the seminal plasma α-L-fucosidase; panel B: output pattern for the sperm-associated α-L-fucosidase. The smaller peaks indicate the $(M+2H)^{2+}$ and $(2M+H)^{+}$ ionized species, respectively.

plasma membranes were identical, thus confirming that the enzyme is located on the plasma membrane. The enzyme prepared from seminal plasma differed in subunit MW and glycosylation features. The seminal fluid enzyme resembles the majority of mammalian fucosidases (2) since it is glycosylated and has a MW of 56 kDa. On the contrary, the plasma membrane fucosidase subunit has a mass of 68 kDa, which is above the range typical of the mammalian enzyme, and does not bear glycosydic moieties recognizable by both ConA and PNA lectins. A difference between seminal plasma and plasma membrane fucosidases was also found in their pH dependence, since the sperm membrane enzyme showed a sharper decrease in activity at high pH. This may be to a modulation of the activity by the glycosidic moieties or to the fact that different amino acid residues contribute to the active site.

It has long been known that glycosidase activities are high in the male genital tract of most animals, but the function of these enzymes is still unknown. Some of these enzymes were recently reported to be associated with the sperm plasma membrane and postulated to be directly involved in sperm-egg interaction in mammals (5,10) and invertebrates (for reviews see 21,22). However, α-mannosidase (23) and α-fucosidase (5) have been biochemically and immunologically char-

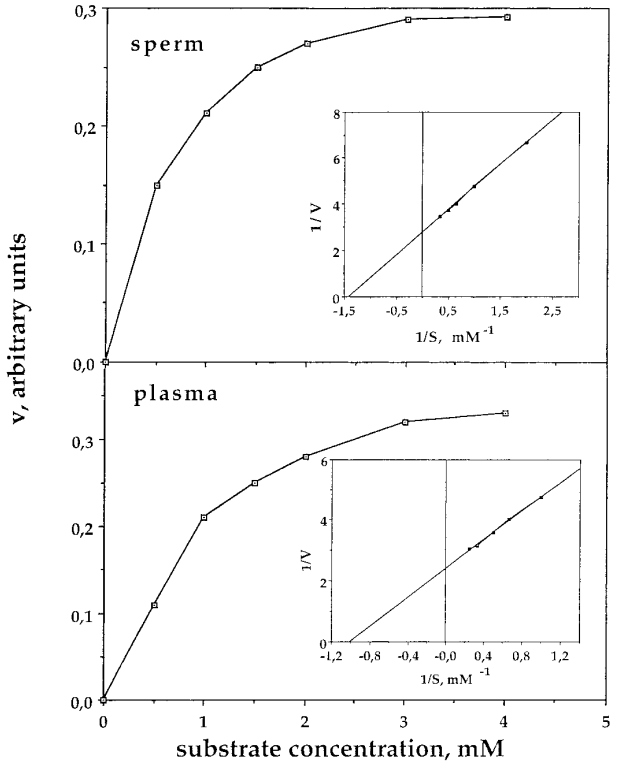

FIG. 4. Dependence of the activity of *Unio elongatulus* α-L-fucosidase on substrate concentration. Panel A: sperm-associated α-L-fucosidase; panel B: gonad α-L-fucosidase. Buffer: 0.1 M McIlvaine buffer, pH 5.0. T: 40 °C. Other assay conditions as described in Methods.

acterized as a single enzyme, present in the reproductive tract and on the sperm surface. In *Unio*, a fucosidase isoform completely different from that present in the surrounding fluid, seems to be expressed and specifically bound to the sperm plasma membrane. Since the ligand for sperm-egg interaction in this species is known and available in sufficient quantities for biochemical studies, the *Unio* model is valuable for a detailed investigation of the mechanism of glycosidase-substrate interaction during fertilization.

ACKNOWLEDGMENTS

We thank Prof. V.D. Vacquier, Scripps Institution of Oceanography, UCSD, for helpful discussions on the manuscript. This research has been supported by grants (40-60%) from the Italian Ministry of the University and of Science and Technology (MURST).

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