# Characterization of Sea Urchin Sperm Membrane Proteins which Interact with a Major Acrosome Reaction-Inducing Substance, Fucose Sulfate Glycoconjugate

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# Characterization of Sea Urchin Sperm Membrane Proteins which Interact with a Major Acrosome Reaction-Inducing Substance, Fucose Sulfate Glycoconjugate

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**ABSTRACT**—Intact sea urchin spermatozoa were successfully biotinylated with NHS-LC-Biotin and the biotinylated spermatozoa retained the viability. Analysis of the membrane prepared from the biotinylated spermatozoa of the sea urchin *Hemicentrotus pulcherrimus* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that several proteins such as wheat-germ agglutinin (WGA)-binding protein (220 kDa), guanylyl cyclase (131 kDa), sperm-activating peptide-I (SAP-I)-crosslinking protein (71 kDa), GPI-anchored protein (63 kDa) and functionally unknown proteins (50 kDa and 30 kDa) were specifically biotinylated. Experiments using spermatozoa of sea urchins, *Anthocidaris crassispina* and *Clypeaster japonicus* showed that several proteins similar to those of *H. pulcherrimus* spermatozoa were also labeled with NHS-LC-Biotin.

Fucose sulfate glycoconjugate (FSG) isolated from the jelly coat of *H. pulcherrimus* was mixed with solubilized biotinylated sperm membrane proteins of *H. pulcherrimus*, *A. crassispina* or *C. japonicus* and then subjected to gel filtration chromatography on a Sepharose 2B column, indicating that only two biotinylated *H. pulcherrimus* sperm proteins were coeluted with *H. pulcherrimus* FSG.

## INTRODUCTION

For successful fertilization, spermatozoa must locate the egg, penetrate the egg's extracellular matrix and bind to and fuse with the egg plasma membrane. When exposed to the extracellular matrix (jelly coat) of the unfertilized sea urchin eggs, sea urchin spermatozoa undergo the acrosome reaction in which the acrosomal vesicle is exocytosed and an acrosomal process of filamentous actin is extended from the tip of the sperm head (Dan, 1967). The jelly coat is mainly composed of two large acidic glycoconjugates and oligopeptides (Garbers *et al.*, 1982; Garbers *et al.*, 1983; Hotta *et al.*, 1970; Isaka *et al.*, 1970; Ishihara *et al.*, 1973; SeGall and Lennarz, 1979; Suzuki *et al.*, 1981; Suzuki, 1989) and has been shown to activate sperm metabolism (Christen *et al.*, 1983; Epel, 1978; Ohtake, 1976) and induce the acrosome reaction (Collins and Epel, 1977; Dan, 1952, 1954, 1956; Decker *et al.*, 1976; Kinsey *et al.*, 1979; Kinsey *et al.*, 1980; Lopo and Vacquier, 1980; SeGall and Lennarz, 1979; Summers and Hylander, 1975; Summers *et al.*, 1975; Tilney *et al.*, 1973). The acrosome reaction of sea urchin spermatozoa is accompanied by ionic changes which cause an increase in the intracellular pH and trigger plasma membrane depolarization. In the last 15 years, substances responsible for activation of sperm metabolism were intensively studied and found to be oligopeptides. These oligopeptides are called sperm-activating peptides. Sperm-activating peptide-I (SAP-I, GFDLNGGGVG) is the first oligopeptide isolated from the egg jelly coats of *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus* (Garbers *et al.*, 1982; Suzuki *et al.*, 1981). SAP-I induces a number of physiological and biochemical events in sea urchin spermatozoa such as 1) a transient elevation of intracellular levels of cAMP and cGMP (Garbers *et al.*, 1982) and $Ca^{2+}$ (Hoshino *et al.*, 1992; Schackmann and Chock, 1986), and 2) a transient activation of the membrane form of guanylyl cyclase (Bentley *et al.*, 1986). It also induces a proton efflux across the sperm plasma membrane, resulting in an increase in the intracellular pH (Hoshino *et al.*, 1992; Repaske and Garbers, 1983; Schackmann and Chock, 1986). In addition to the above, the peptide has been shown to promote the acrosome reaction in *H. pulcherrimus* spermatozoa as a specific co-factor of a major acrosome reaction-inducing substance, fucose sulfate glycoconjugate (FSG) (Yamaguchi *et al.*, 1989). Prior to the induction of these physiological and biochemical events, the peptide seems to bind to specific receptors on the sperm plasma membrane (Smith and Gabers, 1983). SAP-I is known to crosslink specifically to a 77 kDa protein in *S. purpuratus*

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spermatozoa (Dangott *et al.*, 1989) or to a 71 kDa protein in *H. pulcherrimus* spermatozoa (Shimizu *et al.*, 1994).

FSG, a large acidic glycoconjugate in the jelly coat, has been shown to be the natural inducer of the acrosome reaction (SeGall and Lennarz, 1979; Garbers *et al.*, 1983; Shimizu *et al.*, 1990; Keller and Vacquier, 1994). It increases cAMP concentration, activates a cAMP-dependent protein kinase and stimulates $Ca^{2+}$-accumulation by spermatozoa. However, information about the sperm surface molecules which may interact with FSG is limited although several sperm surface glycoproteins have been reported to be involved in the induction of the acrosome reaction (Podell and Vacquier, 1985; Trimmer *et al.*, 1987). In *S. purpuratus* spermatozoa, several glycoproteins (320 kDa, 210 kDa, 140 kDa, 80 kDa and 60 kDa) were accessible to radioiodination of intact spermatozoa as well as isolated sperm membrane vesicles (Lopo and Vacquier, 1980; Podell *et al.*, 1984; Trimmer *et al.*, 1987). In *H. pulcherrimus* spermatozoa, several proteins such as WGA-binding protein (220 kDa), guanylyl cyclase (131 kDa for phosphorylated form and 128 kDa for dephosphorylated form) and sperm-activating peptide I-crosslinking protein (71 kDa) have been suggested to locate on the sperm surface (Sendai and Aketa, 1991; Harumi *et al.*, 1991, 1992; Shimizu *et al.*, 1994).

To obtain a deeper understanding of the sperm surface proteins which are involved in sperm-egg interaction, we attempted to label and characterize the sperm surface proteins (Hardy and Garbers, 1994). Here, we report that several new proteins in addition to proteins mentioned above were biotinylated and two proteins (63 kDa and 50 kDa) showed specific interaction with FSG.

## MATERIALS AND METHODS

*Animals*

Sea urchins, *H. pulcherrimus*, *Anthocidaris crassispina* and *Clypeaster japonicus* were collected near Noto Marine Laboratory of Kanazawa University.

*Chemicals*

Sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-Biotin) and disuccinimidyl suberate were products of Pierce (Rockford, IL, USA). ECL Western blotting detection system, Hybond-C-super membrane, streptavidin biotinylated horseradish peroxidase complex, anti-mouse Ig (biotinylated species-specific whole antibody from sheep) and anti-rabbit Ig (biotinylated species-specific whole antibody from donkey) were purchased from Amersham-Japan (Tokyo, Japan). Biotinylated standard proteins (low and high ranges) for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Prestained SDS molecular weight markers and raw wheat germ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). GGGY-SAP-I (GGGY-GFDLNGGGVG) was synthesized for us at National Institute for Basic Biology, Okazaki, Japan. $Na^{125}I$(3.7GBq/ml NaOH solution, pH 10) was obtained from Du Pont/New England Nuclear (Boston, MA, USA). 3-[(3-cholamidopropyl)-dimethylammnio]-1-propanesulfonate (CHAPS) was a product of Dojindo Chemical Institute Co. (Kumamoto, Japan).

*Antibodies*

Rabbit anti-serum against a synthetic peptide (KPPPQKLTQEAIEVAANRVIPDDV) corresponding to the C-terminal amino acid sequence of *S. purpuratus* sperm guanylyl cyclase was generously provided by Dr. T. Quill in Professor David L. Garbers' laboratory (University of Texas Southwestern Medical Center, Dallas, Texas, USA). A monoclonal antibody against a 63 kDa sperm protein (GPI-anchored protein) of *S. purpuratus* was a kind gift of Professor Victor D. Vacquier (Scripps Institution of Oceanography, University of California, San Diego, CA, USA) (Mendoza *et al.*, 1993). Rabbit anti-serum against *H. pulcherrimus* sperm creatine kinase was made in our laboratory (Harumi *et al.*, 1992).

*Purification of* H. pulcherrimus *FSG*

*H. pulcherrimus* eggs were collected in filtered sea water after intracoelomic injection of 0.5 M KCl. The egg suspension was adjusted to pH 5.0 with 0.1 N HCl to dejelly and the dejellyed eggs were allowed to sink. The supernatant was centrifuged at 10,000 xg for 20 min at 4°C, and the resulting supernatant (jelly solution) was stored at -20°C until use.

FSG was purified from the jelly solution by sequential chromatography on a Sepharose 2B column as reported previously (Shimizu *et al.*, 1990). A frozen and thawed jelly solution was centrifuged at 15,000 xg for 30 min at 4°C. The precipitate was dissolved in deionized and distilled water (DDW), followed by the addition of an equal volume of 0.2 M NaCl. The mixture was applied to a Sepharose 2B column (5 x 86 cm) equilibrated with 0.1 M NaCl and eluted with 0.1 M NaCl at a flow rate of 50 ml/hr at 4°C. Fractions containing fucose were pooled, concentrated using an ultrafiltration apparatus with a YM-10 Diaflo membrane (Amicon Division, W. R. Grace of Co. MA, USA) and centrifuged at 10,000 xg 30 min at 4°C. The resulting supernatant was stored at -20°C until use.

*Biotinylation of sea urchin spermatozoa*

Spermatozoa obtained by intracoelomic injection of 0.5M KCl were collected as "dry sperm" at room temperature and stored on ice until use. Usually the dry sperm were used for experiments within 24 hr. Dry sperm (4 ml) were suspended in 40ml of Buffer A (0.5 M NaCl, 1 mM EDTA and 50 mM HEPES, pH 7.5) and centrifuged at 130 xg for 2 min to remove cell debris. The spermatozoa in the supernatant were pelleted by centrifugation at 4,000 xg for 30 min at 4°C and then suspended in 40 ml of Buffer A. The suspension was mixed with 0.5 ml of freshly prepared 25 mM NHS-LC-Biotin and incubated at 20°C for 1 hr with gentle shaking. The suspension, after being mixed with 0.5 ml of freshly prepared 25 mM NHS-LC-Biotin, was incubated at 20°C for 1 hr with gentle shaking and then 0.25 ml of 1 M Tris-HCl (pH 8.0) was added to terminate the reaction. After a 10 min-incubation at 20°C, the sperm suspension was centrifuged at 4,000 xg for 30 min at 4°C. The resulting sperm pellet was suspended in 40 ml of Buffer A, centrifuged at 4,000 xg for 30 min at 4°C, resuspended in 10 ml of Buffer A and centrifuged at 12,000 xg for 20 min at 4°C. The pellet (biotinylated spermatozoa) was used for immediate experiments or otherwise stored at -70°C until use.

*Fertilization of* H. pulcherrimus *eggs with biotinylated spermatozoa*

*H. pulcherrimus* eggs were collected in filtrated sea water after intracoelomic injection of 0.5 M KCl. A 5% washed egg suspension was fertilized in artificial sea water (ASW; 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2 \cdot 2H_2O$, 27.1 mM $MgSO_4 \cdot 7H_2O$, 4.4 mM $NaHCO_3$) using spermatozoa which were incubated with or without NHS-LC-Biotin for varying time periods. At 2 min after sperm addition, 100 eggs were randomly taken and numbers of eggs with or without the fertilization envelope were counted under a microscope. Fertilization rates (%) were calculated from the results of three independent experiments using the same batch of eggs and spermatozoa.

*Preparation of sperm membranes*

Unfrozen biotinylated or non-biotinylated spermatozoa were suspeneded in 20 volumes of ASW containing 2 mM benzamidine-HCl, 0.01% (w/v) streptomycin, 0.01% (w/v) penicillin G and 20 mM Tris-HCl (pH 9.0) (Suzuki *et al.* 1987). The suspension was incubated with gentle agitation for 12 hr at 4°C and then centrifuged at 8,000 xg for 30 min at 4°C. The supernatant was saved and re-centrifuged under the same conditions. The resulting supernatant was then centrifuged at 100,000 xg for 60 min to pellet the sperm membranes. The pellet was resuspended in an appropriate volume of ASW and stored at -70°C until use.

*Isolation of WGA-binding protein*

The 220 kDa WGA-binding protein was isolated from the membranes of non-biotinylated spermatozoa by the method reported previously (Shimizu *et al.*, 1994).

*Gel filtration of biotinylated sperm proteins with FSG*

Biotinylated sperm membranes prepared as described above were suspended in solubilization buffer (1% CHAPS and 20 mM Tris-HCl pH 8.2 in 2-fold concentrated ASW) and subjected to sonication for 30 sec on ice. The homogenate was centrifuged at 4°C for 1 hr at 100,000 xg and the supernatant (biotinylated sperm protein solution) was stored on ice until use.

Five ml of *H. pulcherrimus* FSG solution (227 μg protein/ml) was mixed with an equal volume of biotinylated sperm protein solution (170 μg protein/ml) and allowed to stand for 30 min on ice. The mixture was then applied to a Sepharose 2B column (2.5 x 85 cm) equilibrated with a buffer (0.1% CHAPS and 10 mM Tris-HCl, pH 8.2 in ASW). Proteins were eluted with the equilibration buffer at a flow rate of 20 ml/hr and fractions of 5 ml were collected. After determination of the protein concentration of each fraction, an aliquot (1 μl) of each fraction was spotted on a Hybond-C-super membrane and biotinylated proteins were detected by ECL. An equal volume of each fraction was also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One of the two equivalent gels was silver-stained to detect proteins and the other one was analyzed by ECL Western blotting. For control purposes, the sample containing only *H. pulcherrimus* FSG or only biotinylated sperm proteins was subjected to gel filtration on the same Sepharose 2B column.

*SDS-PAGE and Western blotting*

SDS-PAGE was carried out essentially as described by Laemmli (1970) using a 5-15% linear gradient gel for analysis of biotinylated sperm proteins and a 6% gel for analysis of FSG. Proteins in the gel were visualized by silver-staining (Morrissey, 1981). For ECL Western blotting, proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane (Amersham Hybond C-super) using a Multiphor II Electrophoresis System (Pharmacia LKB Biotechmology) and a transfer buffer [39 mM glycine, 48 mM Tris , 0.0375%(w/v) SDS and 20% (v/v) methanol ] at 4°C for 60-120 min at constant current (0.8 mA/cm$^2$). The nitrocellulose membrane was blocked with TBS-T [137 mM NaCl, 0.1%(v/v) Tween 20 and 20 mM Tris-HCl, pH7.6] containing 5% skim milk at 4°C for overnight, washed twice with TBS-T, and was shaken in 15 ml TBS-T once for 15 min and twice for 5 min. The membrane was then incubated with streptavidin biotinylated horseradish peroxidase complex solution diluted 1:5000 with TBS-T for 1 hr at room temperature and washed with TBS-T once for 15 min and twice for 5 min. Finally, the membrane was incubated with streptavidin biotinylated horseradish peroxidase complex solution diluted 1:5000 with TBS-T for 1 hr at room temperature and washed with TBS-T once for 15 min and four times for 5 min. The proteins that reacted with antibody were located by ECL Western blotting according to the manufacturer's instructions. After transferring of proteins, the SDS-gel was silver-stained to confirm whether the transferring was completed.

*Crosslinking of GGGY($^{125}$I)-SAP-I to* H. pulcherrimus *spermatozoa*

GGGY-SAP-I was iodinated by the chloramine-T method using radioactive sodium iodide and purified as described previously (Yoshino and Suzuki, 1992). Sperm membranes prepared from 16.7 mg wet weight of spermatozoa were incubated with GGGY($^{125}$I)-SAP-I (10 pmol) in 1 ml of ASW buffered with 10 mM HEPES (pH 8.2) for 10 min at 20°C. The reaction was stopped by the addition of ice-cold ASW (0.9 ml, 10 mM HEPES, pH 8.2), and the mixture was centrifuged at 15,000 xg for 5 min at 4°C. The resulting pellet was resuspended in ASW (90 μl, 10 mM HEPES, pH 8.2) and incubated with a crosslinking reagent, disuccinimidyl suberate (1 mM) in dimethylsulfoxide (10 μl) for 30 min at 20°C. The incubation was terminated by the addition of ASW (0.9 ml, 10 mM Tris, pH 8.2) and then 0.5 ml of 30% (w/v) trichloroacetic acid was added.

*Determination of protein concentration*

The concentration of protein was determined by the Lowry method (1951) modified by Schacterle and Pollack (1973) using bovine serum albumin as a standard.

## RESULTS

*Purification of* H. pulcherrimus *FSG*

The precipitate obtained by centrifugation of frozen-thawed egg jelly solution contained almost entire FSG presented in the original solution. Approximately 15 mg of FSG proteins were obtained from one time chromatography of the resolubilized precipitate fraction which contained approximately 20 mg proteins. As reported previously, when the purified FSG was analyzed by SDS-PAGE in the presence of 2-mercaptoethanol two major protein bands (258 kDa and 237 kDa) and one minor red-stained protein band (120 kDa) were detected. However, these three components could not be detected in SDS-PAGE without 2-mercaptoethanol.

*Biotinylation of sea urchin sperm proteins*

Spermatozoa of three different species of sea urchins (*H. pulcherrimus*, *A. crassispina* and *C. japonicus*) were biotinylated as described in Methods, and the sperm membranes were prepared by the nitrogen cavitation. The sperm membrane proteins were analyzed by SDS-PAGE and subsequent ECL Western blotting. As shown in Fig. 1, several biotinylated proteins were detected in samples from spermatozoa of these species.

*Viability of biotinylated spermatozoa*

As shown in Table 1, *H. pulcherrimus* spermatozoa treated with NHS-LC-Biotin for 120 min retained the fertilizing ability although the motility of the spermatozoa began to decline at 60 min after the treatment. The eggs fertilized with the biotinylated spermatozoa developed normally at least to the blastula stage. When the spermatozoa were incubated with NHS-LC-Biotin for 4 hr the fertilizing ability was decreased to 80% of zero time control spermatozoa.

*Identification of* H. pulcherrimus *biotinylated sperm membrane proteins*

When biotinylated *H. pulcherrimus* sperm membrane proteins were analyzed by SDS-PAGE, one biotinylated protein with 220 kDa was electrophoresed to the same position of the WGA lectin-binding protein purified from *H. pulcherrimus*

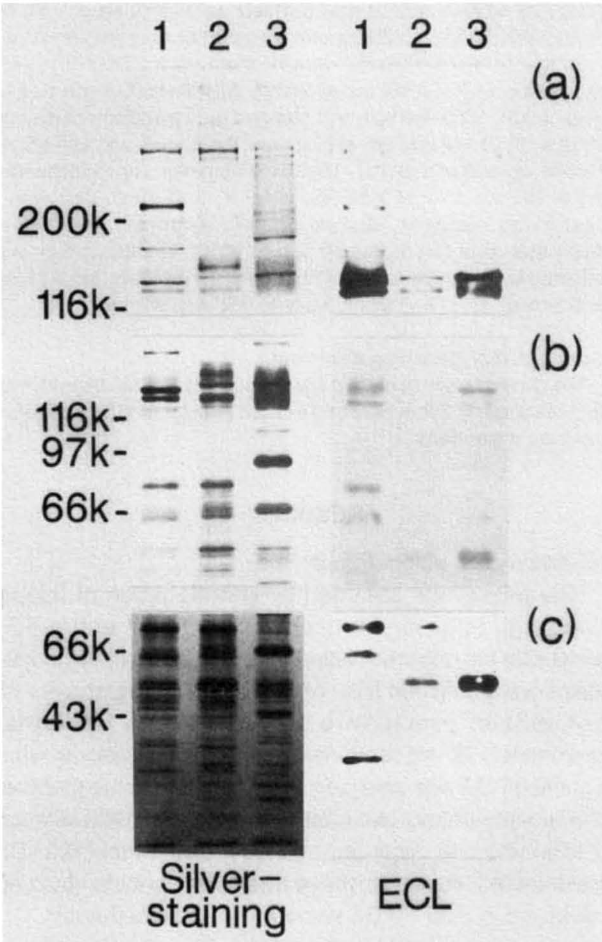


Fig. 1. SDS-PAGE and subsequent ECL Western blot analysis of biotinylated sperm proteins. Membrane proteins (5 µg) were prepared from biotinylated spermatozoa of *H. pulcherrimus* (lane 1), *A. crassispina* (lane 2) or *C. japonicus* (lane 3) and subjected to SDS-PAGE using a 5-15% linear gradient gel. The proteins in the gel were silver-stained (left panel) or transferred electrophoretically onto a Hybond-C-super membrane at 0.8 mA/cm$^2$ for 120 min (a), 60 min (b) and 30 min (c) and then located by ECL (right panel).

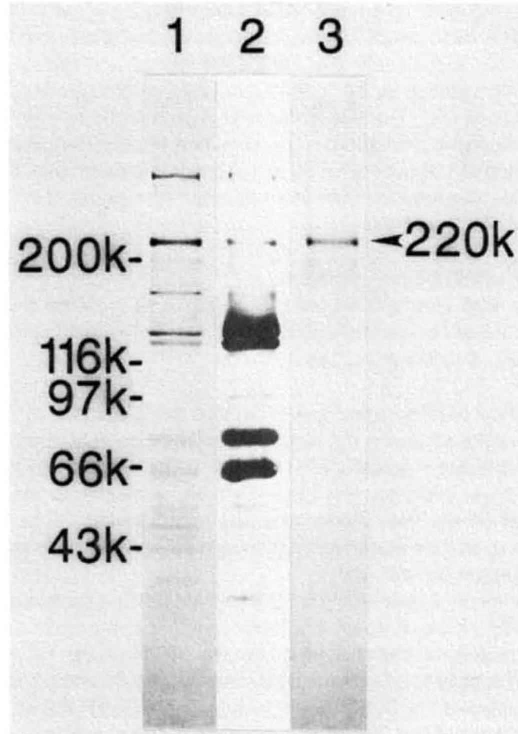


Fig. 2. Identification of WGA-binding proteins by SDS-PAGE and subsequent ECL Western blot analysis. Proteins (5 µg) prepared from non-biotinylated (lane 1) or biotinylated spermatozoa (lane 2) and purified WGA-binding protein (0.5 µg) (lane 3) were separated by SDS-PAGE using a 5-15% linear gradient gel. The gel was cut into two pieces and one piece of the gel was analyzed by ECL-Western blotting (lane 1 and lane 2) and the other one was silver-stained (lane 3).

Table 1. The viability of biotinylated spermatozoa

| Biotinylation time (min) | Fertilized eggs (%, n=3) by biotinylated spermatozoa | by nonbiotinylated spermatozoa |
|---|---|---|
| 0 | 98.3 | 100 |
| 30 | 99.3 | 100 |
| 60 | 99.0 | 99.3 |
| 90 | 98.3 | 99.3 |
| 120 | 96.0 | 95.0 |

The viability of biotinylated spermatozoa was checked on the fertilization ability

Table 2. Major biotinylated proteins in *H. pulcherrimus* spermatozoa

| Sizes | Fractions | Identified proteins |
|---|---|---|
| 220 kDa | Membrane fraction | WGA-binding protein |
| 137 kDa | Membrane fraction | Tail creatine kinase |
| 131 kDa | Membrane fraction | Guanylyl cyclase |
| 71 kDa | Membrane fraction | SAP-I-crosslinking protein |
| 63 kDa | Membrane fraction | GPI-anchored protein |
| 50 kDa | Membrane fraction | Unknown |
| 30 kDa | Membrane fraction | Unknown |

spermatozoa (Fig. 2). Similarly, an other biotinylated protein was electrophoresed to a position which corresponds to the molecular mass (71 kDa) of a $^{125}$I-GGGY-SAP-I-crosslinking protein (Fig. 3). Subsequently other biotinylated *H. pulcherrimus* sperm membrane proteins were separated by SDS-PAGE using a 5-15% linear gradient gel and then analyzed by ECL Western blotting. Three biotinylated proteins (137 kDa, 131kDa and 63 kDa) reacted with antibodies specific for *H. pulcherrimus* sperm tail creatine kinase, *S. purpuratus* sperm guanylyl cyclase or *S. purpuratus* GPI-anchored protein (Fig. 3). Major biotinylated proteins in *H. pulcherrimus* spermatozoa are summarized in Table 2.

*Gel filtration of biotinylated sperm proteins with* H. pulcherrimus *FSG*

When proteins solubilized from membranes prepared by

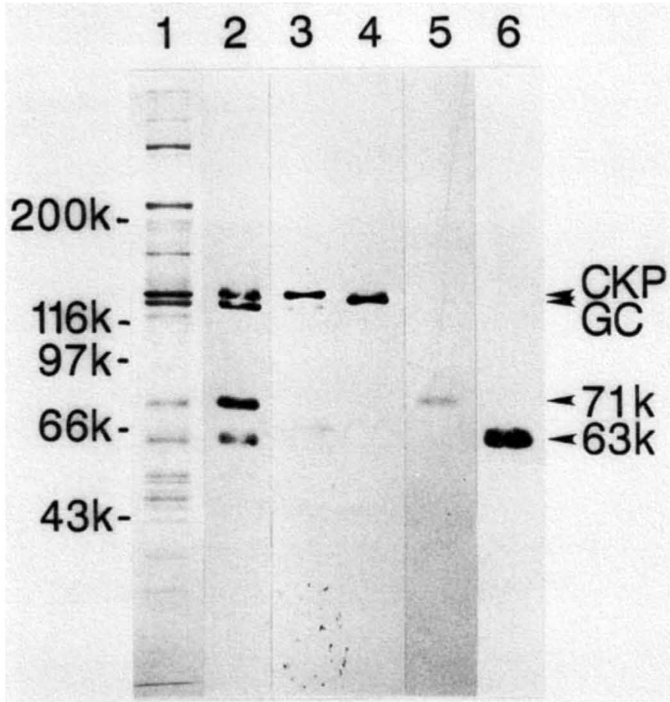


Fig. 3. SDS-PAGE and Western blot analysis of *H. pulcherrimus* sperm membrane proteins. Sperm membrane proteins (2.5 μg) prepared from biotinylated (lanes 1 and 2) or non-biotinylated (lanes 3, 4, and 6) spermatozoa were subjected to SDS-PAGE using a 5-15% linear gradient gel. The proteins in the gel were silver-stained (lane 1) or transferred onto a Hybond-C-super membrane and located by ECL without antibody (lane 2) or using anti-*H. pulcherrimus* sperm creatine kinase rabbit anti-serum (lane 3), site-directed anti-*S. purpuratus* guanylyl cyclase rabbit anti-serum (lane 4) and a monoclonal antibody against a 63 kDa protein of *S. purpuratus* spermatozoa (lane 6). Sperm membranes were cross-linked with GGGY($^{125}$I)-SAP-I in the presence of disuccinimidyl suberate and solubilized in 10% (v/v) SDS. The proteins (10 μg) were then subjected to SDS-PAGE, followed by autoradiography (lane 5).

hypotonic treatment of *H. pulcherrimus* biotinylated spermatozoa were combined with *H. pulcherrimus* FSG and subjected to chromatography on a Sepharose 2B column, only 63 kDa and 50 kDa biotinylated proteins were coeluted with FSG (Fig. 4). On the other hand, when *H. pulcherrimus* biotinylated sperm membrane proteins alone were applied to the column, almost all biotinylated proteins including 63 kDa and 50 kDa biotinylated proteins were eluted after the expected peak fraction of FSG. Co-elution of proteins solubilized from biotinylated sperm membranes of *C. japonicus* or *A. crassispina* with *H. pulcherrimus* FSG was not detected except weak signals by dot blot analysis.

## DISCUSSION

Prior to the induction of the acrosome reaction, a major acrosome reaction-inducing substance in sea urchin egg jelly, FSG, must interact specifically with sperm surface substance(s). In this sense, it is important to have knowledge of sperm surface proteins. In the present study, we demonstrated that in *H. pulcherrimus*, several sperm proteins such as WGA-binding protein, membrane form of guanylyl cyclase, SAP-I-crosslinking protein and GPI-anchored protein, all of which have been demonstrated to be plasma membrane proteins, can be labeled by incubation with NHS-LC-Biotin, and the biotinylated spermatozoa retained the viability for at least 120 min. This suggests that biotinylation using NHS-LC-Biotin is a useful technique for labeling sperm surface proteins. Biotinylation has several advantages over radioiodination to labele sperm surface proteins: 1) the reaction time is much shorter than radiolabeling and the resulting biotinylated proteins can be easily detected by ECL Western blotting with high sensitivity and 2) biotinylated spermatozoa or sperm membranes prepared from them can be stored in a freezer at -40°C for a long time (at least 1 year) without any problems in further characterization and analysis of the biotinylated proteins.

Of the biotinylated *H. pulcherrimus*, *A. crassispina* and *C. japonicus* sperm proteins, only two proteins (63 kDa and 50 kDa) from *H. pulcherrimus* spermatozoa were coeluted with *H. pulcherrimus* FSG in gel filtration chromatography. This sugests that these proteins have specific interaction with *H. pulcherrimus* FSG. To characterize these proteins further, we are currently isolating these proteins from the biotinylated *H. pulcherrimus* spermatozoa by affinity chromatography using anti-biotin agarose column.


## ACKNOWLEDGMENTS

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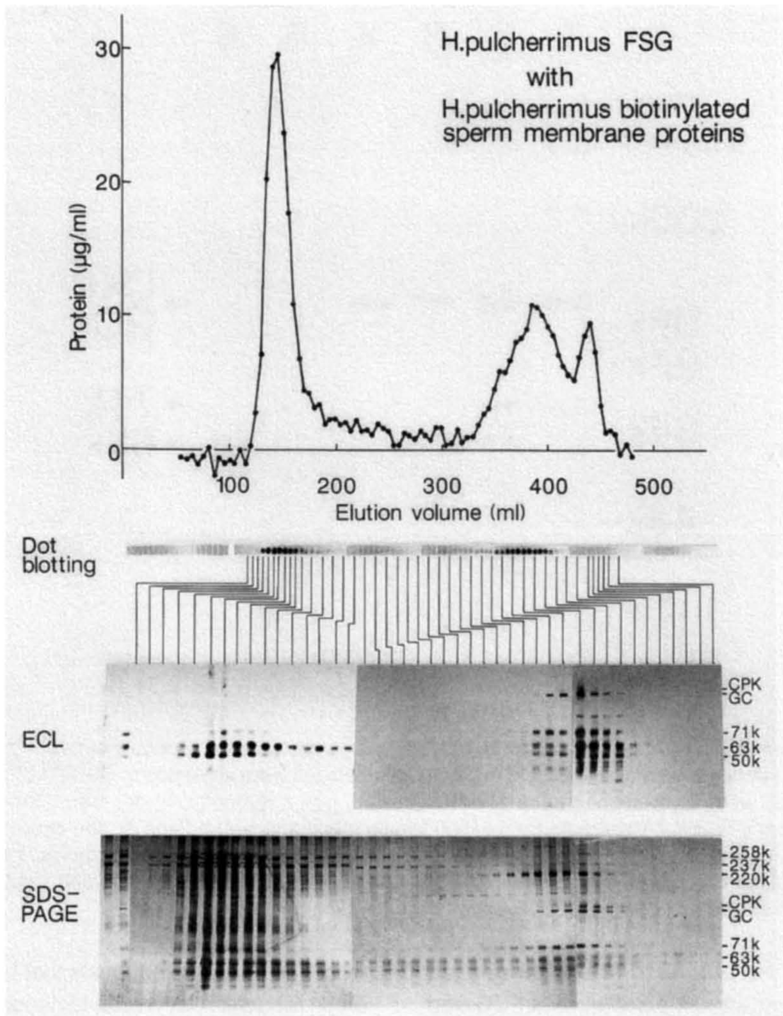


Fig. 4. Gel filtration profile of *H. pulcherrimus* FSG with solubilized biotinylated sperm membrane proteins on a Sepharose 2B column. *H. pulcherrimus* FSG (1385 μg protein) and biotinylated *H. pulcherrimus* sperm proteins (850 μg) were combined and the mixture was subjected to chromatography on a Sepharose 2B column equilibrated with ASW containing 0.1% CHAPS and 20 mM Tris, pH 8.2. Proteins were eluted with equilibration buffer (20 ml/hr at 4°C) and fractions of 5 ml were collected. An aliquot (1 μl) of each fraction was spotted on Hybond-C-super membrane and detected by ECL. An equal volume of each fraction was subjected to SDS-PAGE using a 5-15% linear gradient gel. The proteins in the gel were silver-stained (lower) or transferred onto Hybond-C-super membrane and located by ECL (upper).

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