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THE ISOLATION AND CHARACTERIZATION OF A CONCANAVALIN A RECEPTOR FROM BOAR SPERMATOZOA SURFACE

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Summary

Boar spermatozoa were radioactively labeled by either lactoperoxidase-catalysed iodination or galactose oxidase oxidation followed by reduction with tritiated sodium borohydride. Plasma membrane glycoproteins were solubilized with the non-ionic detergent Nonidet P40 and separated by affinity chromatography on concanavalin A-Sepharose. A major water-soluble concanavalin A receptor of molecular weight greater than 160 000 was isolated by gel filtration and ion-exchange chromatography. Its amino acid and carbohydrate composition were determined. This glycoprotein is susceptible to digestion by trypsin or chymotrypsin.

Introduction

The plasma membrane plays an important role in the maturation and capacitation of spermatozoa and in the fertilization of the egg [1]. Several surface modifications involving the proteins and glycoproteins of the membrane occur during these biological processes. Despite considerable ultrastructural and immunological investigations, little is known about the chemical structure and function of these components [2]. Lectin labeling and binding were used as molecular probes in investigations of the externally oriented carbohydrates of the membrane. Edelman and Millette [3] have shown that concanavalin A agglutinates mammalian spermatozoa, which indicates that glycoprotein receptors for this lectin are present on the cell surface. On mouse spermatozoa, the number of receptors has been estimated as being $5 \cdot 10^7$ per cell. Labeling with fluorescent concanavalin A revealed that these receptors were mainly located in the acrosomal region. Using agglutination experiments, Nicolson and co-wor-

kers [4,5] have demonstrated the presence of binding sites for several lectins on rabbit and hamster sperm surface. Due to scarcity of the material, these studies have not led to the isolation of any spermatozoa plasma membrane glycoproteins.

The boar is an advantageous source of large quantities of sperm (300—500 ml, 10^8 cells per ml). This easily accessible starting material gives the opportunity for biochemical studies of membrane components on a preparative scale.

In this study we have radioactively labeled the surface proteins of boar spermatozoa, purified a concanavalin A receptor by affinity chromatography and characterized its general chemical properties.

Materials and Methods

Materials

Concanavalin A was purchased from Serva (Heidelberg, F.R.G.). Sephacryl S-200 and Sepharose 4B were from Pharmacia (Uppsala, Sweden), CM-52 cellulose from Whatman (U.K.).

Lactoperoxidase was obtained from Boehringer (Mannheim, F.R.G.), galactose oxidase from Sigma (St. Louis, MO, U.S.A.). Trypsin and chymotrypsin were from Worthington (Freehold, NJ, U.S.A.). Other chemical reagents were from commercial sources.

Radioactive reagents were obtained from the Commissariat à l'Energie Atomique (Saclay, France) or Amersham-France.

Cells

The boar semen was collected at the Station de Physiologie Animale, Institut National de la Recherche Agronomique, Jouy en Josas (France). It was filtered through gauze. The spermatozoa were centrifuged at low speed ($1000 \times g$) and were separated from the seminal plasma. The cells were washed three times with phosphate-buffered 0.15 M NaCl, pH 7.2. Aliquots were resuspended in the same buffer and labeled with radioactive reagents within 3 h after collection.

Methods

Preparation of a concanavalin A-Sepharose column. The procedure of Lloyd was used [6]. Approx. 7 mg concanavalin A were attached per ml settled Sepharose 4B beads. The immobilized lectin was equilibrated in a 20 mM sodium acetate buffer/0.15 M NaCl, pH 7.2, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM in phenylmethylsulfonyl fluoride (elution buffer).

Radioactive labeling of the spermatozoa membrane proteins. Lactoperoxidase-catalysed radioiodination was performed on aliquots ($(1—3) \cdot 10^8$ cells) by the method of Morrison [7] with 2 mCi $Na^{125}I$. After labeling, the cells were washed three times with phosphate-buffered 0.15 M NaCl, pH 7.2, and once with phosphate-buffered 0.15 M KI, pH 7.2.

Galactose and galactosamine residues of the surface glycoproteins were labeled with tritiated borohydride after oxidation by galactose oxidase according to the procedure of Gahmberg and Hakomori [8] as described by Baumann and Doyle [9].

Lysis of spermatozoa. The lysis was carried out for 1 h at 4°C by suspend-

ing the cells and vigorously shaking them in a 20 mM sodium acetate/0.15 M NaCl buffer, pH 7.2, containing 4% Nonidet P40. This buffer was made 20 mM in phenylmethylsulfonyl fluoride to prevent proteolysis by acrosomal proteases. The suspension was then frozen overnight and thawed. It was centrifuged for 30 min at 13 000 × *g*. The supernatant contained the solubilized membrane proteins.

Gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed following the method of Weber and Osborn [10] using 10% acrylamide/0.135% bisacrylamide concentration. All samples were denatured for 5 min at 100°C in a 3% SDS/5% mercaptoethanol solution, pH 6.8. The procedure of Laemmli was also used [11]. The gels were stained with Coomassie brilliant blue R-250. Densitometric recording of the slab gels were performed with a microdensitometer (Joyce and Loebl, U.K.) at 540 nm.

Two-dimensional electrophoresis of membrane proteins was achieved according to the procedure of O'Farrell [12]. In the second dimension, a 15% acrylamide/0.087% bisacrylamide slab gel was used.

Radioactivity measurements. Tritium was counted in a Packard Tri-carb liquid scintillation spectrometer model 3320 with Unisolve I (Koch-Light, U.K.) as scintillator. A γ-counter (model Gamma CG-30, Intertechnique, France) was used for ^{125}I measurements. Autoradiograms of dried slab gels were obtained with Kodirex films (Kodak) after suitable exposure. They were scanned under the same conditions as above.

Amino acid analysis. Amino acid analysis was performed on a Beckman Multichrom B with an ICAP Integrator calculator using a single column procedure [13]. The samples were hydrolyzed in vacuo in 6 M HCl for 18 h at 110°C. The tryptophan content was determined after hydrolysis with 4 N methanesulfonic acid at 110°C for 20 h using a short column of the Beckman apparatus [14]. Methionine and cysteine were determined as methionine sulfone and cysteic acid, respectively, after performic acid oxidation [15].

Carbohydrate analysis. The procedure of Clamp and co-workers was used [16,17]. The samples (0.5 mg) were subjected to methanolysis by 1 M HCl in dry methanol at 85°C for 24 h. The acid was neutralized with silver carbonate. Acetic anhydride was then added and the samples were kept at room temperature for 24 h. The precipitate was centrifuged and extracted twice with methanol. The supernatants were taken to dryness and silylated with the silylating agent described by Clamp during 30 min at room temperature. The silylated monosaccharides were identified on a Hewlett-Packard type 5710 A gas chromatography. An SE-30 4% Chromosorb AW-DMCS column was used. Mannitol was used as an internal standard.

Proteolytic and chemical hydrolysis. The purified iodinated receptor (0.5 mg) was dissolved in 0.1 ml 0.1 M ammonium bicarbonate and digested with trypsin or chymotrypsin. The ratio of enzyme to substrate was 1 : 50 (w/w). Aliquots were withdrawn after 15 min, 1, 3 and 16 h, frozen and lyophilized.

Cleavage of methionyl bonds followed the procedure of Gross [18]. The protein (0.5 mg) was dissolved in 0.1 ml 70% formic acid and treated with 0.5 mg CNBr at room temperature for 18 h. The reaction mixture was then diluted 10-fold with water and lyophilized.

Results

Radioactive labeling of spermatozoa

In independent experiments, approximately 0.5 ml ($1.5 \cdot 10^8$ cells) of cellular pellet was resuspended in 2 ml phosphate-buffered 0.15 M NaCl, pH 7.2, and incubated with 2 mCi $Na^{125}I$ or with 25 mCi tritiated borohydride after galactose oxidase oxidation.

The cells were observed under a microscope and were found intact as judged from the eosine-nigrosine exclusion test [19]. However, the motility of the flagellum disappeared during the labeling procedure. All further purification steps were identical for both radioactive samples. We will only describe the purification of the radioiodinated concanavalin A receptor.

Solubilization of the membrane proteins, electrophoretic analysis of the lysate

The radioactively labeled cells were centrifuged at $1000 \times g$, mixed with 15 ml of spermatozoa pellet ($3 \cdot 10^{10}$ cells), resuspended in 20 ml of the 4% Nonidet P40 solution and treated as described in the Materials and Methods section. The lysed cells were re-extracted once in identical conditions.

An aliquot of the radioiodinated material (10^6 cpm) was withdrawn prior to the dilution with unlabeled cells, washed twice with phosphate-buffered 0.15 M NaCl, pH 7.2, lysed and subjected to two-dimensional electrophoresis according to O'Farrell [12] as shown in Fig. 1. Several labeled membrane proteins differing in size and charge were separated. A highly radioactive protein (indicated by an arrow in Fig. 1) was observed. Its isoelectric point was close to 6.5 and its molecular weight was greater than 160 000.

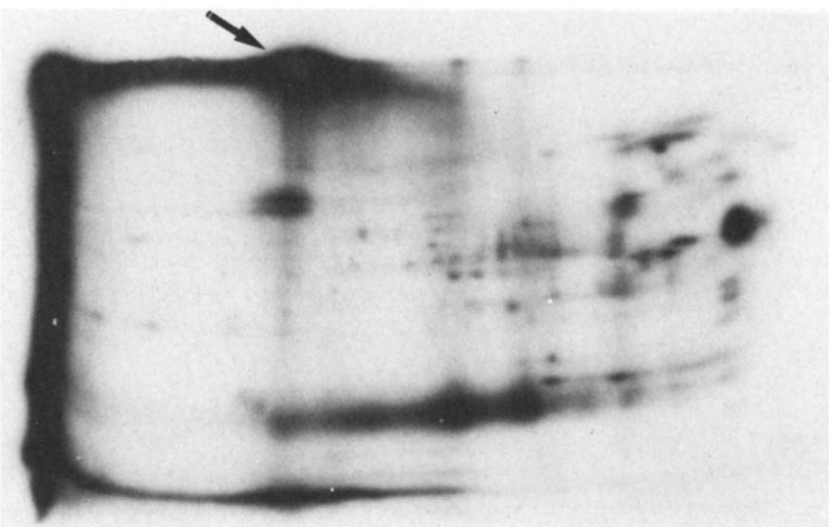

Fig. 1. Two-dimensional electrophoresis of the ^{125}I-labeled proteins from boar spermatozoa plasma membrane. Isoelectric focusing in the horizontal dimension. The pH gradient was 7.5—4.5 from left to right. In the vertical dimension: SDS-polyacrylamide gel electrophoresis, from top to bottom. The arrow indicates the concanavalin A receptor. The autoradiogram was exposed for 3 days.

Affinity chromatography on concanavalin A-Sepharose column

An aliquot (20 ml) of the detergent extract was diluted twice with the elution buffer and loaded on a concanavalin A-Sepharose column (20 × 2.8 cm). The column was washed with the elution buffer until the absorbance decreased to 0.1. The concanavalin A-binding proteins were then eluted with a 0.1 M solution of methyl α-D-mannoside in the same buffer.

Approximately 82% of the ^{125}I radioactivity was recovered in the breakthrough peak (fraction I), about 10% was displaced with the 0.1 M methyl α-D-mannoside solution (fraction II), less than 2% was eluted by increasing the molarity of the saccharide to 0.25 M.

Upon rechromatography of fraction I in the same conditions, almost no lectin-bound protein could be recovered. Fraction II, containing the concanavalin A-binding proteins, was concentrated from 300 ml to 10 ml by ultrafiltration using an Amicon UM-10 membrane. The solution remained clear after concentration.

Sephacryl S-200 chromatography

An aliquot of concentrated fraction II (5 ml) was filtered on a Sephacryl S-200 column as described in Fig. 2. A high molecular weight radioiodinated fraction which contained 17% of the total recovered material and 45% of the radioactivity was eluted first. Two other low radioactive fractions were separated. They were not studied further.

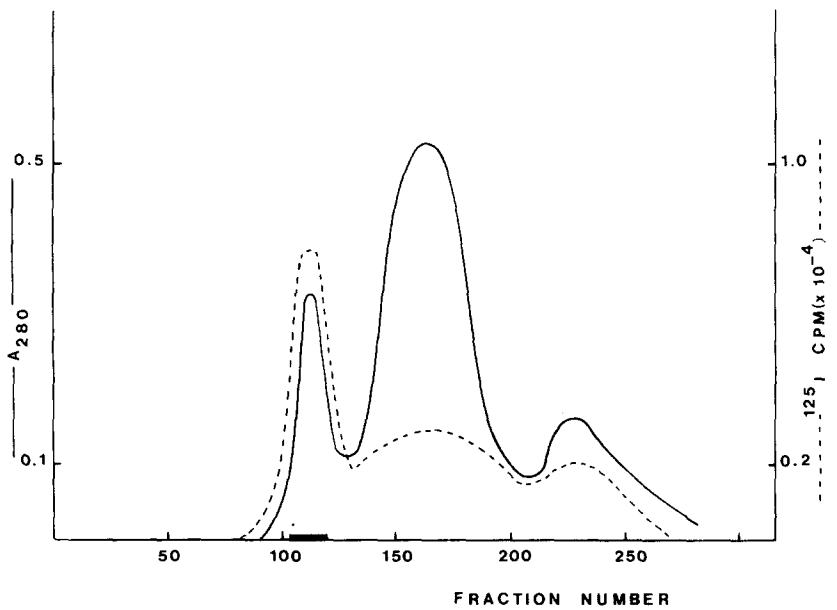

Fig. 2. Sephacryl S-200 chromatography of the concanavalin A receptors. Column: 115 × 2.8 cm. Elution with a 20 mM sodium acetate/0.15 M NaCl buffer, pH 7.2. Fraction: 2 ml/5 min. The horizontal bar indicates the fractions collected.

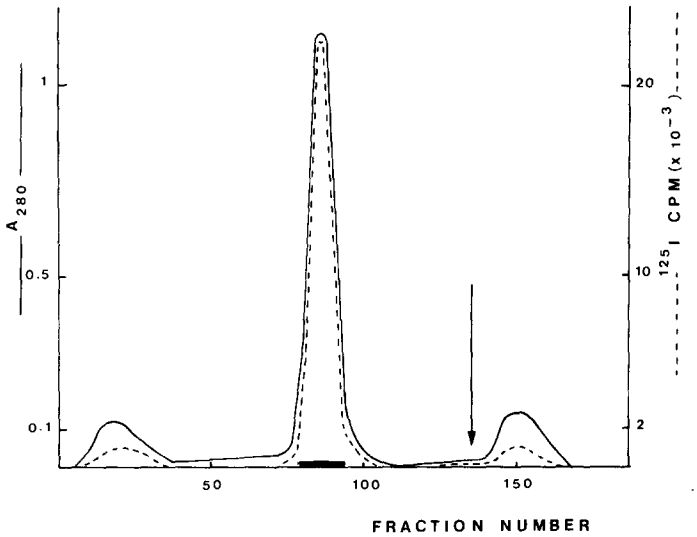

Fig. 3. CM-52 cellulose chromatography of the partially purified concanavalin A receptor. Column: 12 × 1.8 cm. Elution with a linear gradient of 50 mM monosodium phosphate, pH 4.6, to 50 mM disodium phosphate, pH 8.4, followed by 1 M NaCl in 50 mM disodium phosphate as indicated by the arrow. Fraction: 2 ml/5 min. The horizontal bar indicates the fractions collected.

Carboxymethyl-cellulose chromatography

The first fraction eluted from the Sephacryl S-200 column was dialysed against 50 mM sodium monophosphate pH 4.6, then applied to a column of CM-52 cellulose equilibrated in the same buffer and eluted as indicated in Fig. 3. The main radioactive fraction was collected, dialysed against water and lyophilised. The yield of this chromatographic step was 60% as calculated from radioactivity measurements.

In a typical experiment using 100 ml of boar semen (10^{10} cells), 15 mg of radioiodinated concanavalin A receptor was obtained after three purification steps.

In order to isolate the concanavalin A receptor labeled with ^{3}H, the same purification procedure was used. The elution patterns of the three chromatography steps were identical to those found with the iodinated material.

Electrophoretic analyses

The purity of both radioactively labeled receptors was examined on polyacrylamide gel electrophoresis using the procedure of Weber and Osborn [10]. The ^{3}H-labeled receptor gave a single band of an apparent molecular weight greater than 160 000. All the ^{3}H radioactivity was associated with this band (Fig. 4). Attempts to obtain a faster migration of the protein by lowering the concentration of acrylamide resulted in a diffuse band. In this gel system, the iodinated receptor gave a similar result.

The purity of the radioiodinated receptor was further studied in a 5—15% polyacrylamide gradient using the buffer system of Laemmli. A single radioactive band associated with the Coomassie blue staining was observed (see Fig. 5a).

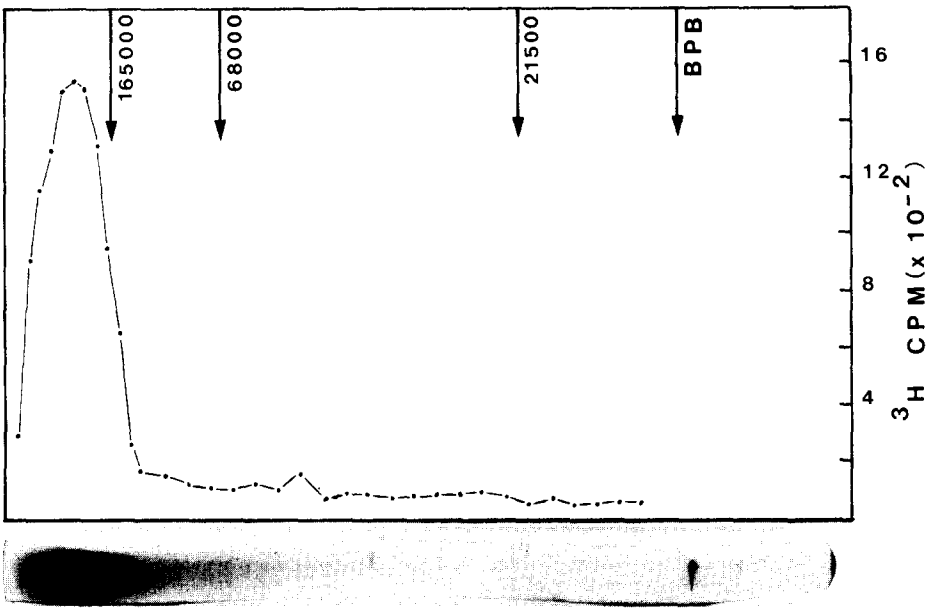

Fig. 4. Gel electrophoresis of the tritiated concanavalin A receptor. Gel system of Weber and Osborn [10]. An unfixed gel was cut in 2-mm slices which were eluted with a 0.1% SDS solution and counted. Standards were RNA polymerase (M_r, 165 000, 39 000), serum albumin (M_r, 68 000) and soybean trypsin inhibitor (M_r, 21 500). BPB, bromophenol blue used as tracking dye.

The purified receptor migrated as a single spot in two-dimensional electrophoresis. Its position on the gel corresponded to the strongly radioactive protein indicated by an arrow in Fig. 1.

Amino acid and carbohydrate composition

The amino acid composition of both tritiated and iodinated receptors was determined. An average value is given in Table I. No tryptophan was detected

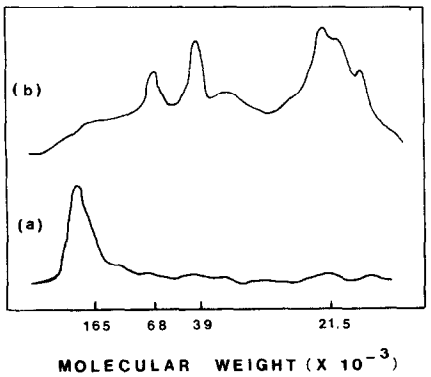

Fig. 5. Gel electrophoresis of the radioiodinated concanavalin A receptor (a) and of its CNBr digest (b). Gel system of Laemmli [11]; 5—15% polyacrylamide gradient. Top of the gel on the left. Densitometric recording of the autoradiogram after 4 days exposure. Same standards as in Fig. 4.

TABLE I

AMINO ACID COMPOSITION OF THE CONCANAVALIN A RECEPTOR

Residues/100 residues		Residues/100 residues	
Asp	8.8	Met **	1.1
Thr	10.5	Ile	4.9
Ser	7.6	Leu	5.8
Glu	11.0	Tyr	4.9
Pro	9.1	Phe	2.2
Gly	8.2	His	1.8
Ala	4.4	Lys	3.4
Cys *	2.1	Arg	4.9
Val	9.1	Trp ***	0

* Determined as cysteic acid.
** Determined as methionine sulfone.
*** Determined after 4 N methane sulfonic acid hydrolysis.

after methane sulfonic acid hydrolysis. This negative result may be due to the presence of carbohydrates which reduce the recovery of this amino acid [14]. The presence of glucosamine and traces of galactosamine were observed in the amino acid analysis.

The following carbohydrate composition (in nM/mg of protein) was obtained: Gal, 248; GlcNAc, 193; GalNAc, 35; Man, 33; NeuNAc, 0. The ratio of carbohydrate to protein was 10% (w/w).

Specificity of binding to concanavalin A

Equal amounts of purified radioiodinated receptor were incubated for 18 h at room temperature with 1-ml aliquots of concanavalin A-Sepharose. After centrifugation, the supernatants were separated and three washes were performed with a 20 mM sodium acetate/0.15 M NaCl/buffer, pH 7.2. The bound receptor was then eluted with 5 ml of a solution of various saccharides dissolved in the same buffer or with 1 M NaCl. This elution was repeated four times. The eluates were pooled and the ^{125}I radioactivity was counted. When solutions of 1 M NaCl or 0.1 M rhamnose, fucose, galactose were used, respectively 15%, 13%, 12%, 18% of the total radioactivity was eluted. This yield increased to 53% when the elution was performed with a 0.1 M methyl α-D-mannoside solution.

The concanavalin A receptors of boar seminal plasma

We have examined the seminal plasma in order to determine whether the concanavalin A receptor that we have isolated from the spermatozoa surface is also present in soluble form.

Using the identical experimental conditions as described above, 50 ml of freshly collected seminal plasma were made 1 mM in phenylmethylsulfonyl fluoride and filtered on the same column of concanavalin A-Sepharose. A fraction was retained on the column and eluted with a 0.1 M methyl α-D-mannoside solution. Approximately 360 mg of bound material was recovered, as estimated from absorbance measurement. A part of this material (100 mg) was concen-

trated and chromatographed on the same Sephacryl S-200 column, that had been used previously.

No appreciable amount (<5%) of high molecular weight proteins was present. We therefore conclude that the membrane concanavalin A receptor is virtually absent from the seminal plasma.

Proteolytic and chemical hydrolysis

The time course digest of the purified iodinated receptor by trypsin or chymotrypsin was analysed by polyacrylamide gel electrophoresis. A 5—15% slab gel gradient was used. In both cases, the original receptor disappeared after 15 min of reaction. Broad and diffuse bands were observed in the low molecular weight region of the gel (data not shown).

The CNBr digest of the iodinated receptor was analysed by polyacrylamide gel electrophoresis as described in Fig. 5b. Two large peptides of molecular weight 60 000 and 35 000 and a group of small peptides were generated.

Discussion

Very few components of mammalian spermatozoa membrane have been purified to a pure state and in quantities that allow biochemical analysis [20—23]. In this paper we describe a high molecular weight surface glycoprotein obtained by affinity chromatography on concanavalin A-Sepharose.

This protein is strongly labeled in situ by the lactoperoxidase-catalysed radioiodination. It is therefore exposed on the cell surface. It appears as a major component in the two-dimensional electrophoresis of the detergent-solubilized membrane proteins.

As it contains galactose, it is also accessible to external labeling with tritium according to the method of Gahmberg and Hakomori [8] as described by Baumann and Doyle [9]. In a control experiment we have found that the boar seminal plasma did not contain any significant amount of a similar high molecular weight concanavalin A receptor.

Further evidence for its location on the cell surface was given by immunofluorescence studies which indicate that this glycoprotein is situated on the entire surface of the boar spermatozoa. Similar antigenic determinants were found on the surface of human spermatozoa (Hermann, J. and Soriano, L., unpublished results).

In order to liberate the glycoproteins from the cell surface, we have used a non-ionic detergent (Nonidet P40). This mild solubilizing agent produces a selective extraction of loosely bound membrane components. A similar observation was made by Keil-Dlouha et al. [24] in the study of surface proteins derived from embryonal carcinoma cells. We therefore suggest that the concanavalin A receptor is a peripheral membrane protein as defined by Singer [25] and not an integral membrane protein the solubilization of which would require strong denaturing conditions including ionic detergents, urea or chaotropic agents.

The detergent extract was fractionated by affinity chromatography on a concanavalin A-Sepharose column. As previously observed [26] the presence of Nonidet P40 did not significantly affect the binding of glycoproteins when a

large excess of lectin is used. The elution of the column was performed with a detergent-free solution of methyl-α-D-mannoside. In these experimental conditions, only the water-soluble concanavalin A receptors were recovered.

The sugar composition of the glycoprotein shows that it is almost devoid of mannose but contains *N*-acetylglucosamine residues which are known as weaker ligands of concanavalin A than mannose or glucose [27]. The specificity of binding to the lectin suggests that these residues are located in terminal position in the glycosidic chain. The low content of *N*-acetylgalactosamine and the high content of serine and threonine indicate that the carbohydrate residues are probably attached to the polypeptidic chain by an *O*-glycosidic linkage, but only a detailed structural study of the saccharide moiety will bring the direct information.

The molecular weight of the concanavalin A receptor is greater than 160 000 but it cannot be accurately determined with the electrophoretic mobility data since the protein migrates as a large band. The presence of carbohydrates may also modify its electrophoretic behaviour. However the CNBr digestion of the protein generates several small peptides and two large peptides (M_r, 60 000 and 35 000) which appear as sharp bands on the gel elctrophoresis. This result is consistent with the high molecular weight mentioned above.

The biological significance of this surface glycoprotein remains unknown.

Since it is present on the entire surface of the spermatozoa, it may stabilize the plasma membrane during the transit of the cell in the female tract. We have observed that the receptor is water-soluble and easily digestible without denaturation and reduction by trypsin or chymotrypsin in contrast to many proteins and glycoproteins which are resistant to proteolytic enzymes. During the capacitation process, this glycoprotein may be progressively removed from the surface by proteases present in the uterus secretions [28]. This hypothesis is corroborated by the observation of Gordon et al. [29] who have shown that in the capacitated rabbit spermatozoa the ability of the acrosomal region to bind concanavalin A was diminished.

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