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Journal of Chromatography B, 746 (2000) 141–150

JOURNAL OF CHROMATOGRAPHY B

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Inhibition of acrosine-like protease activity by a lectin affinity chromatographic bovine seminal plasma fraction containing the PDC-109 and aSFP proteins

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Received 19 August 1999; received in revised form 22 May 2000; accepted 23 May 2000

Abstract

These studies showed that the fractionation of bovine seminal plasma based on lectin agarose affinity chromatography, employing lectins specific to asparagine linked oligosaccharides, and a lectin specific for fucosylated glycans, lead to products with an inhibitory effect on the acrosine-like protease activity. This effect decreases when glycocompounds containing fucosylated Lewis^x structures are removed, suggesting that these compounds might have some role in the modulation of this activity in the bull. In the fraction devoid of high mannose, hybrid and non-bisecting lactosaminic oligosaccharide-containing glycocompounds, PDC-109 and aSFP proteins were detected and characterized at microscale.

Keywords: Acrosine-like protease; Enzymes; PDC-109 protein; aSFP protein

1. Introduction

Seminal plasma is a very complex fluid rich in many types of macromolecules involved in fertilization. Fertility-associated proteins were described in this fluid, as well as glycoproteins which participate in sperm–egg recognition, modulation of capacitation of the spermatozoon, the acrosome reaction, etc., [1–4]. *N*-Linked glycans modulate the physicochemical properties of the proteins to which they are attached, by altering solubility, surface charge and adhesive properties. The most important role of *N*-glycans, however, is the regulation of protein conformation. As a result, they can modulate the biological activity and immunological properties of enzymes, hormones, receptors and lectins. In the rat, for example, *N*-linked glycans regulate the enzymatic activity of plasminogen activator by modulating the conversion of single-chain to double-chain forms and also limit their susceptibility to serum and tissue-derived protease inhibitors [5]. The role of *N*-glycans in fertilization differs among species [6]. Mouse ZP3

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00308-X

(mZP3) is a glycoprotein that functions as primary ligand and inducer of the acrosome reaction [7]. In pigs, the release of *N*-linked carbohydrate chains from ZP3 zona protein (pZP3) markedly reduces its sperm receptor activity [8]. In humans, the biological activity of the M_r 74 000 glycoprotein is lost if either *N*-linked or *O*-linked oligosaccharides are removed [5,9]. On the other hand the presence of fucosylated *N*-glycans was demonstrated in the human spermatozoon [10] and it was proved that the 3-fucosyl *N*-acetyllactosamine (CD15) Lewis^x structures are involved in human sperm–egg interaction [11]. Recently, it was reported that these structures might even have some role in interspecific fertilization [12].

To study the role of *N*-glycans in bull fertility and considering that the most convenient method for the detection of sperm glycans is that of lectin binding [5], we applied lectin affinity chromatography to fractionate bovine seminal plasma by serially removing its *N*-glycan-containing glycoproteins.

The presence of the major acidic bovine seminal plasma proteins BSP-A_1, BSP-A_2 (both known as PDC-109) ([13]; and references therein) and of the aSFP factor [14] all of which are involved in fertilization through the modulation of sperm capacitation and other physiological properties [15,16], was investigated in the purified fractions. The proteins BSP-A_1 and BSP-A_2 differ in the degree of glycosylation. Both are identical in amino acid composition and sequence [13,17]. These proteins interact with lipids [17] and participate in lipid transport. They interact with HDL and together with this lipoprotein they modulate sperm capacitation [15,17]. aSFP belongs to the spermadhesin family which are carbohydrate-binding lectins. They act as sperm-associated binding molecules for zona pellucida glycocompounds and contain the CUB domain. This is a 100–110-residue spanning extracellular module named after the three proteins where it was first recognised: complement subcomponents (Clr/Cls), embryonic sea urchin protein (Uegf), and bone morphogenetic protein 1 (Bmp1). Thirty-one copies of this domain have been identified in 16 functionally diverse proteins, many of which are known to be involved in developmental processes [18,19].

Besides, since the acrosine-like protease activity can be correlated with male fertility [20], analysis of the effects of each fraction on this activity were performed.

2. Experimental

2.1. Starting material

Semen samples were obtained from Holando Argentina bulls with an artificial vagina and centrifuged (6000 *g*, 15 min, 5°C). The supernatant (seminal plasma) was recovered and centrifuged at 10 000 *g* for 60 min at 5°C and freeze–dried until use.

2.2. Acrosine-like protease sample

The acid extract of the epididymal spermatozoa was obtained from bull cauda spermatozoa as reported before ([20]; and references therein).

2.3. Isolation of the seminal plasma fractions by lectin affinity chromatography

The procedure employed for the fractionation of the bovine seminal plasma is outlined in Fig. 1 which shows the different steps.

A Concanvalin A Sepharose (Con A) column 22×1 cm (Amersham Pharmacia, Uppsala, Sweden) was prepared as reported before [21,22]. The bed volume of the column was 25 ml, the flow-rate was 18 ml/h. Aliquots of 1 ml each were collected.

A 400-mg amount of freeze–dried bovine seminal plasma was dissolved in 1 ml of 50 m*M* phosphate buffer, pH 7, 200 m*M* NaCl (PBS) and applied at the top of the column. This was eluted with PBS until the absence of proteins [23] and carbohydrates [24] was confirmed. The interacting material was eluted first with PBS containing 5 m*M* methyl-α-D-glucoside and then with PBS containing 200 m*M* methyl-α-D-mannoside. In the fractions eluted with the inhibitory sugars only proteins were determined [23]. The materials eluted with each buffer were separately combined, dialyzed, freeze–dried and kept at −20°C until use.

Taking into consideration the possibility that very low-molecular-mass products are present, an aliquot

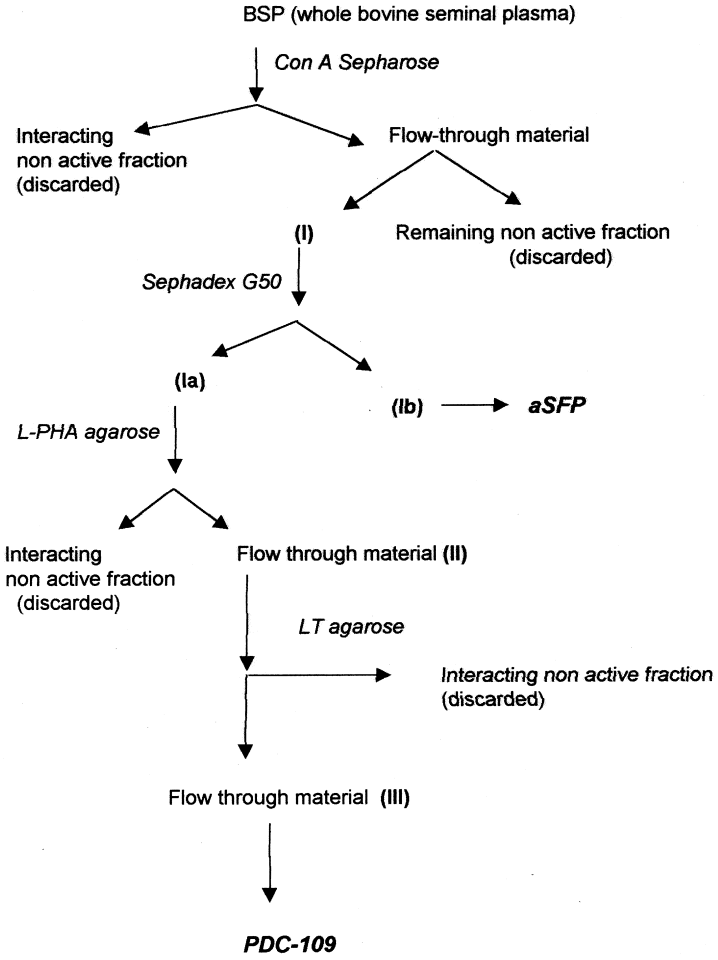

Fig. 1. Flow sheet of the fractional process. A 400-mg amount of freeze–dried bovine seminal plasma was dissolved in 1 ml of 50 m*M* phosphate buffer and applied to the top of the Concanvalin A (Con A) column. The interacting and the flow-through materials were separately pooled, dialyzed, freeze–dried and analyzed for proteins, carbohydrates and inhibitory effect on the acrosine-like protease activity. The active flow-through material (**I**) was chromatographed through a Sephadex G50 column and the fractions obtained (**Ia**) and (**Ib**) separately pooled. The most active fraction was chromatographed through *Phaseolus vulgaris* leucoagglutinin (L-PHA) agarose. The interacting and the flow-through materials were separately pooled. The most active fraction (II) was chromatographed through *Lotus tetragonolobus* (LT) agarose affinity column. In the flow-through material, PDC-109 glycoprotein was obtained.

of the eluted material was dialyzed using small pore dialyzing tubes (M_r 1200 cut-off) and another using M_r 12 000 cut-off dialyzing tubes. The preparative studies were made using the latter tubes in order to isolate molecules of high molecular mass [3,4].

The flow-through material and the interacting fractions were analyzed separately for inhibitory effect on acrosine-like protease activity. The active fraction (**I**) was chromatographed through a Sephadex G50 column and the fractions thus obtained (**Ia**) and (**Ib**) were separately analyzed for inhibitory effects on acrosine-like protease activity [20]. The most active fraction (**Ia**) was chromatographed using a *Phaseolus vulgaris* leucoagglutinin (L-PHA) agarose affinity column.

Phaseolus vulgaris leucoagglutinin agarose column (L-PHA, Sigma, St. Louis, MO, USA) was packed and equilibrated with PBS. The bed volume of the column was 3 ml, the flow-rate was 8 ml/h and 0.5-ml aliquots were collected. The starting

material was 10 mg of fraction (**Ia**) (Fig. 1). The flow-through material was eluted with PBS until the absence of proteins [23] and carbohydrates [24] was confirmed. The retarded compounds tightly bound to the column, were eluted by the following elution sequence: (1) 100 m*M* Tris buffer, pH 8.5, 500 m*M* NaCl; (2) 100 m*M* sodium acetate buffer, pH 4.5, 500 m*M* NaCl; (3) PBS. The materials eluted with each buffer were separately combined, dialyzed, freeze–dried and kept at −20°C until use.

The interacting and the flow-through materials were analyzed separately for inhibitory effects on the acrosine-like protease activity. The active fraction (**II**) was chromatographed using a *Lotus tetragonolobus* (LT) agarose affinity column.

The *Tetragonolobus purpureas* agarose column (*Lotus tetragonolobus*, LT; Sigma) was packed and equilibrated with 10 m*M* Tris buffer, pH 8.0, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$ and 150 m*M* NaCl (TBS). The bed volume of the column was 5 ml, the flow-rate was 14 ml/h and 0.5-ml aliquots were collected.

The starting material was 10 mg of fraction (**II**) (Fig. 1). The flow-through material was eluted with TBS until the absence of proteins [23] and carbohydrates [24], was confirmed. The interacting material was eluted by TBS containing 500 m*M* L-(−)-fucose. The materials eluted with each buffer were separately combined, dialyzed, freeze–dried and kept at −20°C until use.

The interacting and the flow-through fractions were separately pooled, dialyzed, freeze–dried and analyzed for total proteins [23], neutral sugars [24] and inhibitory effect on acrosine-like protease activity. The active fraction (**III**) was submitted to further studies.

2.4. Size-exclusion chromatography

A column of Sephadex G50 Superfine (100×1.5 cm) (Amersham Pharmacia) was used. The Con A interacting fraction was discarded and the peak of the flow-through material [fraction (**I**)] (1 ml of 20 mg/ml solution) was applied to the column (Fig. 1), eluted with distilled water and monitored at 230, 260 and 280 nm.

The standards employed were: aprotinin M_r 6500; cytochrome *c* M_r 12 400; carbonic anhydrase M_r 29 000; bovine serum albumin M_r 66 000; Blue Dextran M_r 2 000 000 (Sigma).

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Denaturing and reducing conditions [25] were used with a discontinuous gel. Resolving gel was 12.5% T, 2% C [T=(g acrylamide+g Bis)/100 ml solution; C=g Bis/% T] in 1 m*M* Tris buffer (1 m*M* Tris with 0.9% glycine and 1% SDS). Running conditions were 100 V in the stacking and 200 V in the resolving gels.

Gels were stained with Coomassie Brilliant Blue or Schiff reagents.

For sequence determination studies the SDS–PAGE was performed in a Mini Protean II system from Bio-Rad. The gel employed and the running conditions were similar to those described before.

2.6. Enzyme assay

Inhibition of acrosine-like protease activity was assayed as described before [20] using *N*-α-benzyl-DL-arginine-*p*-nitroanilide (BAPNA, Sigma) as substrate. The inhibitory effect of each chromatographic peak fraction was tested by adding to the incubation mixture 40 μl of each freeze–dried sample (4 mg/ml). Controls of the incubation mixture without the sample plus an equivalent volume of sample buffer (200 m*M* Tris, pH 8.5) were made.

2.7. Analysis of sugar composition

The sugar composition of the samples (650 μg) was analyzed after hydrolysis with 0.2 *M* trifluoroacetic acid (TFA) (90 min, 120°C) using a Dionex high-performance anion-exchange chromatography (HPAEC) system with a pulse amperometric detector (E_1=0.05 mV, E_2=+0.6 mV, E_3=−0.6 mV [26]). A CarboPac PA-1 (250×4 mm) column was used, eluted isocratically with 15 m*M* NaHO.

2.8. Chemical studies

Proteins were analyzed as described by Lowry et al. [23], neutral sugars by the phenol sulfuric spectrophotometric method [24], sialic acid and phos-

phorous according to Warren [27] and Bartlett [28], respectively.

2.9. "In gel" digestion

After SDS–PAGE the "in gel" digestion was performed according to Rosenfeld et al. [29].

Briefly this was carried out as follows: the band to be digested was cut, introduced into an Eppendorf tube, washed twice with 150 μl of a mixture of acetonitrile–200 m*M* ammonium carbonate (1:1, v/v, pH 8.9) for 20 min at 30°C, dried in a Speedvac evaporator, treated with 5 μl of 200 m*M* (pH 8.9) ammonium carbonate plus Tween 20 (0.02%, v/v) and then digested with 2 μl trypsin (0.25 mg/ml in 200 m*M* ammonium carbonate, pH 8.9).

After the digestion tryptic peptides were separated by reversed-phase high-performance liquid chromatography (HPLC) (Applied Biosystems Model 140A; Foster City, CA, USA) on a Brownlee C_{18} column (220×2.1 mm) equilibrated with 5% (v/v) acetonitrile, 0.1% TFA in water. Elution was performed at a flow-rate of 0.2 ml/min with a 8–48% (v/v) acetonitrile, 0.1% TFA linear gradient in 70 min.

Selected peptides were applied to a polybrene-coated glass filter and sequenced in an Applied Biosystems Model 477 A Automatic Sequencer run according to the manufacturer's instructions.

3. Results

3.1. Chromatographic studies

The Con A Sepharose column (Fig. 2) yielded 25% of the starting material when dialysis of the interacting and the flow-through fractions were separately made using M_r 12 000 cut-off tubing and 92% when M_r 1200 cut-off tubes were employed. The material retained by the Con A Sepharose

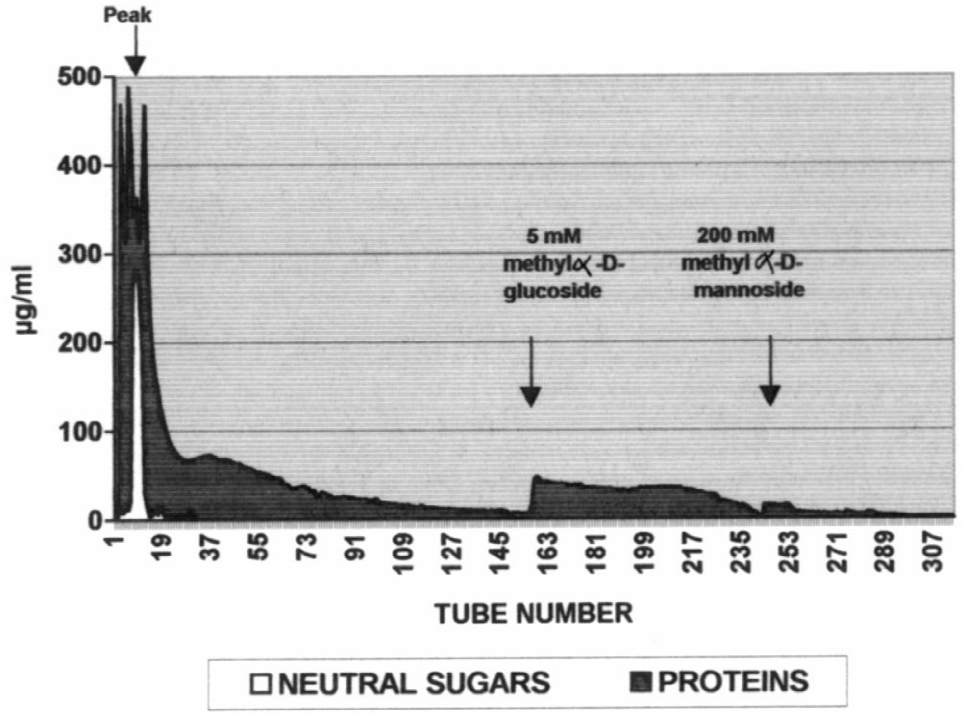

Fig. 2. Affinity chromatography of bovine seminal plasma on Concanvalin A Sepharose (Con A Sepharose) column. A 400-mg amount of freeze–dried bovine seminal plasma in 1 ml of PBS was applied to the Con A column (22×1 cm, bed volume 25 ml). Elution was performed first with PBS followed by PBS containing 5 m*M* methyl-α-D-glucoside and then with PBS containing 200 m*M* methyl-α-D-mannoside at a flow-rate of 18 ml/h. Aliquots of 1 ml were collected.

column did not show any effect on the acrosine-like protease activity and was discarded. The peak of the Con A flow-through fraction (**I**) contains 60% of proteins and 4% of carbohydrates (Table 1). A 40-μg amount of (**I**) inhibited 85% of the acrosine-like protease activity (Table 1). The remaining portion of the flow-through material was not active and was discarded. The gel filtration chromatography of (**I**) (Fig. 1) yielded near 100% [40% of fraction (**Ia**) and 60% of fraction (**Ib**) showing each other slightly different molecular mass]. (**Ib**) was eluted in the position of the ovalbumin (M_r 45 000) and (**Ia**) in that of bovine serum albumin (M_r 67 000). (**Ia**) kept the inhibitory effect of (**I**) on the acrosine-like protease activity while 40 μg of (**Ib**) only inhibited 30% of this activity.

When fraction (**Ia**) was chromatographed through a L-PHA affinity column the bound fraction was not active and the flow-through (**II**) kept the inhibitory effect on the enzymatic activity shown by (**Ia**). Fraction (**Ia**) (Fig. 1) was submitted to L-PHA agarose chromatography. The active flow-through material (**II**) (75% inhibition of acrosine-like protease activity) (Fig. 1) (Table 1) was chromatographed on a LT agarose affinity column and fraction (**III**) was thus obtained (Fig. 1). This fraction showed 32% of inhibitory effect of the acrosine-like protease activity (Table 1).

The affinity chromatography of (**II**) on LT agarose (Figs. 1 and 3) yielded 30% of non active bound material eluted with L-(−)-fucose and was discarded, while the flow-through (**III**) (40 μg) inhibited 32% of the acrosine-like protease activity. These results show that the elimination of *N*-glycans and fucosylated glycans from bull seminal plasma lead to products with acrosine-like protease inhibitory activity but this activity decreases with purification. The flow-through from the LT agarose column retains less than half of the activity of fraction (**II**) (Table 1). The monosaccharides found in fraction (**III**) were *N*-acetylgalactosamine, galactose and glucose (1:1:1) (molar rates). Sialic acid was 0.1% and organic phosphorous 0.4%.

The percent of proteins and carbohydrates of the active fractions are shown in Table 1.

Table 1
Total proteins, neutral sugars and percent of inhibitory effect on the acrosine-like enzymatic activity of the active fractions[a]

Fraction	Proteins[b]	Sugars[c]	% of inhibition[d]
(**I**)	60	4	85
(**Ia**)	84	5	80
(**Ib**)	38	3	30
(**II**)	51	6	75
(**III**)	36	10	32

[a] The active Con A flow-through material (**I**), the fractions (**Ia**) and (**Ib**) obtained from the chromatography of (**I**) through Sephadex G50 column, the L-PHA flow-through material (**II**) and the LT flow-through material (**III**) were separately pooled, dialyzed, freeze–dried and then analyzed for total proteins by the Lowry method [23], neutral sugars by the phenol sulfuric acid method [24] and percent of inhibition of acrosine-like protease activity by the Chen method [20].

[b] mg of proteins per 100 mg of dry material.

[c] mg of neutral sugar per 100 mg of dry material.

[d] Percent of inhibition of the acrosine-like protease activity.

3.2. Analysis by electrophoresis and microsequentation studies

When (**Ib**) was submitted to SDS–PAGE a major band of M_r 14 000 and traces of another of M_r 16 000, were obtained. The ''in gel'' digestion of the major band (M_r 14 000) and microsequentiation of three of the peptides obtained give the following sequences: EVLYFQDPQA; MDWLPR and NTNX-GGILKEESG. These sequences show 100% homology with the portions corresponding to residues 125–134; 21–26 and 27–39, respectively of the amino acid sequence of aSFP protein, thus allowing the identification of (**Ib**) as aSFP protein [14].

Fraction (**III**) showed a major band of about M_r 13 000–14 000 and traces of M_r 25 000 on SDS–PAGE (Fig. 4). The major band was digested ''in gel'' and the peptides were fractionated by HPLC on a C_{18} column. The amino acid sequence of one of the peptides was XYETXTK with a high homology with peptide 104–110 of protein PDC-109 identical to BSP-A_1 and BSP-A_2 [4,30,31]. The sequence of another peptide was IGSMWMSW, showing a 100% homology with residues 111–118 of the same protein.

The flow-through from the LT agarose column (**II**) showed one band in SDS–PAGE.

The active fractions (**I**), (**Ia**), (**II**) and (**III**) (Fig. 4) were stained with Comassie Brilliant Blue or with Schiff reagents showing the presence of glycopro-

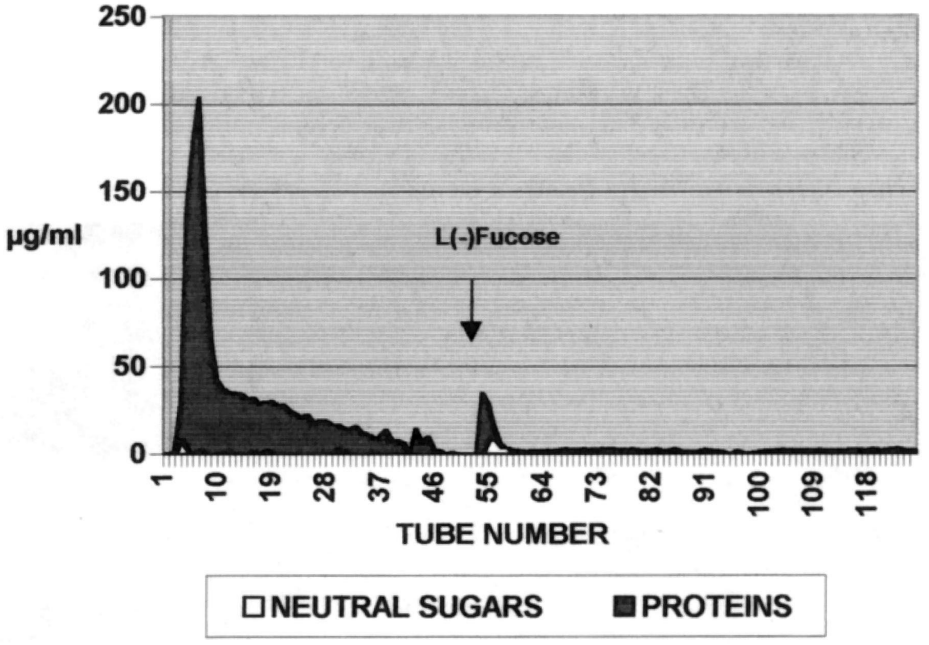

Fig. 3. Affinity chromatography of fraction (**II**) on *Lotus tetragonolobus* agarose (LT agarose) column. A 10-mg amount of fraction (**II**) in 0.5 ml of PBS was applied to the LT column (10×0.75 cm, bed volume 5 ml). Elution was performed first with TBS followed by TBS containing 500 m*M* L-fucose at a flow-rate of 14 ml/h. Aliquots of 0.5 ml were collected.

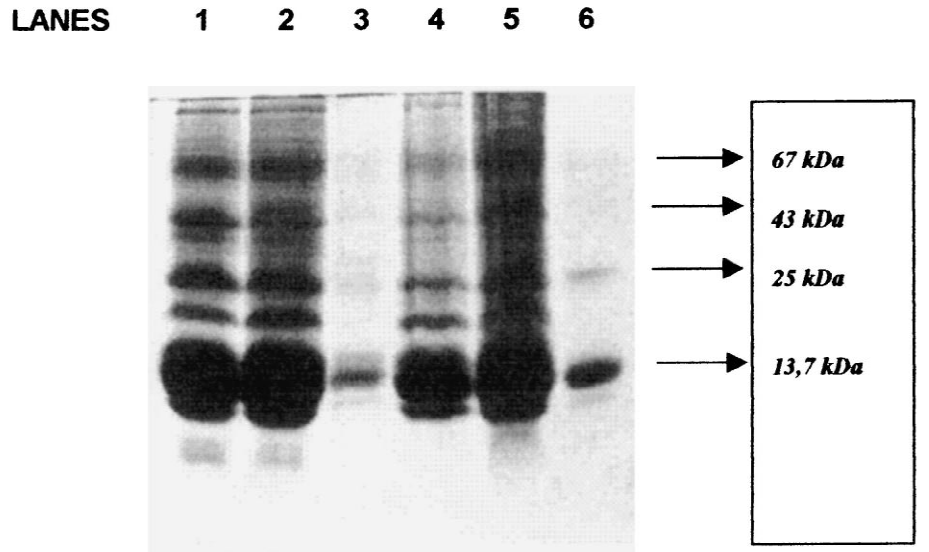

Fig. 4. SDS–PAGE of bovine seminal plasma and the active fractions. Molecular mass standards: bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen A (M_r 25 000), ribonuclease A (M_r 13 700). Lane 1: bovine seminal plasma, lane 2: fraction (**I**), lane 3: fraction (**Ib**), lane 4: fraction (**Ia**), lane 5: fraction (**II**), lane 6: fraction (**III**).

teins. On the other hand fraction (**Ib**) was stained only with Coomassie Brilliant Blue reagent.

4. Discussion

The fractionation of bovine seminal plasma by lectin affinity chromatography using lectins specific for asparagine-linked oligosaccharides, demonstrates, on the basis of the specificities of the lectins employed, that this fluid appears to contain the three types of *N*-oligosaccharide-containing glycocompounds: high mannose, hybrid and complex type [32,33]. However it is known that lectins have no absolute specificity so, on this basis, it is not possible to give more structural details. The very low yield of the Con A Sepharose column when the M_r 12 000 cut-off tubes were used for dialysis, demonstrates the presence of a high percentage of low-molecular-mass compounds. The material eluted from Con A Sepharose with methyl-α-D-glucoside contains biantennary complex-type sugars which bind weakly to Con A, while the high mannose and hybrid type sugar chains, which contain multiple binding sites, strongly bind to the column and are eluted with 0.2 *M* methyl-α-D-mannoside [33,34]. Neither the high mannose, hybrid or biantennary complex type sugar containing fractions have inhibitory effects on the acrosine-like protease activity. The peak of the Con A flow-through fraction (**I**) which is devoid of these structures [33,34], highly inhibits the acrosine-like protease activity, but since this is a very complex fraction, it is not possible to reach any conclusion about the role of the *N*-glycans in it. The elimination of complex *N*-lactosaminic oligosaccharide containing glycocompounds which are retained by the L-PHA agarose column [34], does not decrease the inhibitory effect on enzymatic activity. When fucosylated glycocompounds, which bound to LT agarose [35] are removed, the inhibitory effect on the acrosine-like protease activity decreases, suggesting that these compounds might be involved on this effect, even though other reasons cannot be discarded, i.e., denaturation of the protein(s) contained in this fraction (**III**) or the dissociation of a dialyzable factor from this/these protein(s) during the chromatography on the LT column.

The *Lotus tetragonolobus* lectin has high affinity for oligosaccharide chains containing structure (a) (Fig. 5) or part of this structure or related ones, so the lectin also binds structure (b) (Fig. 5) even though the affinity is four-times lower than for (a). These type of structures are important components of the cellular surfaces and play a role in cell–cell interactions. They appear in type *O*-oligosaccharides (terminal sugar sequences of glycolipids of human blood A, B, H, Lewis and Forsman antigens) and in type *N*-oligosaccharides of glycoproteins ([10]; and references therein). Immobilized LT lectin was used to fractionate complex type *N*-glycans containing the Le^x determinant [35] (structure b) which is present in the sperm surface. CD15 is a family of molecules having in common the pentasaccharide lacto-*N*-fucopentaose III (LNF-III). This pentasaccharide contains the immunodominant trisaccharide determinant (b). It was demonstrated that these determinants are involved in sperm–egg interactions [11]. It seems likely that the elimination of lactosaminic fucosylated glycans decreases but does not suppress the inhibitory enzymatic effect.

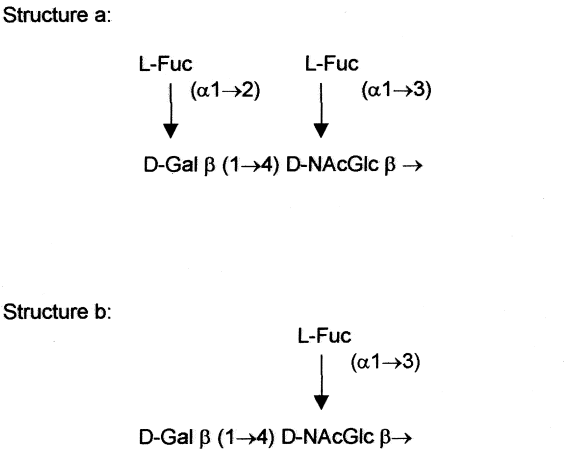

Fig. 5. Fucosylated structures of oligosaccharide chains with affinity towards *Lotus tetragonolobus* lectin.

Since difucosylated diantennary *N*-glycans bind to Con A [33–35], these glycocompounds are not present either in the LT interacting material or in fraction (**III**). On the other hand, highly branched *N*-glycans and trifucosylated triantennary *N*-glycans tightly bound to LT [35] might be included in the interacting fraction which did not show inhibitory effect on acrosine-like protease activity. The

fucosylated glycocompounds of this fraction which might include Le^x antigen [11,35], may participate in some events which involve molecular complementarity or might have another physiological role. From our results it seems that perhaps only certain fucosylated glycocompounds could strengthen, at least ''in vitro'', the inhibitory effects on the acrosine-like protease activity of PDC-109 protein. The inhibitory enzymatic effect decreases from (**II**) to (**III**) suggesting a possible contribution of the Le^x fucosylated glycocompounds to this effect even though they are not active by themselves. Since lipids do not bind the lectins, the lack of increase in the inhibitory activity with each successive column passage suggests that lipids themselves are not responsible of the inhibitory effect of the acrosine-like protease activity.

The presence of PDC-109 glycoprotein in (**III**) and aSFP in (**Ib**) was demonstrated by the 100% homology of the amino acid sequence of the polypeptides analyzed [13,14]. The monosaccharide composition of PDC-109 analyzed by Dionex, agrees with that of the carbohydrate portion of this glycoprotein except for the presence of glucose which might reflect contamination with glycolipids [15,17]. The presence of organic phosphorous confirms this supposition. The detection of sialic acid by the method of Warren [27] is also consistent with the identification of this protein [31].

In conclusion, the results of this work show that the serial elimination of the *N*-linked glycoproteins from bovine seminal plasma is a useful method for the isolation of fractions containing the major bovine seminal plasma proteins (BSP proteins). The fact that these fractions display inhibitory effects on the acrosine-like protease activity is consistent with the capability of BSP proteins to modulate the capacitation of the spermatozoon [15,17]. Besides, this work suggests that this capability might be displayed through the modulation of the acrosine activity and that the aSFP (a spermadhesin) [16] could participate in this modulation.

Acknowledgements

The authors wish to thank the LANAIS-PRO, UBA-CONICET (National Protein Sequencing Facility) Junín 956, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina for the peptide sequencing and to Universidad de Buenos Aires.

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