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Characterization of Anti-Sticking Factor-II From Goat Epididymal Plasma

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A previous study has characterized the major 47 kDa anti-sticking factor (ASF-I) from goat cauda-epididymal plasma (Roy, N., and Majumder, G.C., *Biochim. Biophys. Acta, 991:*114–122, 1989). This study reports the purification and characterization of ASF-II, another anti-sticking factor from the goat epididymal plasma. ASF-II was purified to apparent homogeneity by using concanavalin A–agarose affinity chromatography, DEAE-cellulose chromatography, alumina gel adsorption, and isoelectric focussing techniques. It showed a single protein band by both non-denaturing and SDS–polyacrylamide gel electrophoresis. ASF-II showed a molecular weight of 36,000 and a sedimentation constant of 2.4S. ASF-II is largely stable to heat treatment and it is a specific glycoprotein having high affinity and specificity for its anti-sticking action. At saturating concentration (1 nM) it inhibited adhesion of nearly 50% of spermatozoa to the glass surface of the haemocytometer counting chamber. Both the protein and sugar parts of the factor are essential for the anti-sticking activity since it lost its activity completely when treated with trypsin, L-fucosidase, or mannosidase. ASF-II does not coat the glass surface and it binds to spermatozoa. Data are consistent with the view that ASF-II has not been derived from the larger ASF-I molecule due to its enzymic modifications. Both ASF-I and -II had no effect on sperm forward motility as evidenced by spectrophotometric motility assays, indicating thereby the suitability of the factors to improve the existing sperm motility assays by eliminating the possibility of cell-sticking artifacts.

Key words: anti-sticking factor, spermatozoa, sperm adhesion, goat epididymal plasma

It is well documented that washed spermatozoa stick to the glass surface of a haemocytometer counting chamber and this sperm adhesion to glass may pose as a great artifact in the sperm motility assays [1,2]. Previous studies from this laboratory have provided evidence for the occurrence of anti-sticking activity in goat cauda-EP that strongly inhibits sperm adhesion to glass [2]. More recently the major anti-sticking

Abbreviations: EP, epididymal plasma; ASF, anti-sticking factor; PMSF, phenylmethylsulfonylfluoride; RPS, modified Ringer's solution.

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factor (ASF-I) of EP has been purified to apparent homogeneity and characterized [3]. ASF-I is a heat-stable 47 kDa glycoprotein and it inhibits nearly 60% of the sperm adhesion to glass. It showed high affinity and specificity for its anti-sticking activity. Both the protein and sugar parts of the factor are essential for its ASF activity. It binds specifically to the sperm surface attachment site rather than the glass surface for its anti-sticking action. The present study describes the purification and characterization of another anti-sticking factor (ASF-II) from goat EP.

MATERIALS AND METHODS

Chemicals

DEAE-cellulose, alumina gel (Cγ), α-methyl mannoside, phenylmethylsulfonyl-fluoride (PMSF), L-fucosidase, β-N-acetyl glucosaminidase, α-mannosidase, neuraminidase, β-galactosidase, ovalbumin, and trypsin (2 × crystallized) were obtained from Sigma Chemical Company, St. Louis. Concanavalin A–agarose was a product of Hygro-chemical Ltd., Calcutta. Ampholine (pH 5–8) was purchased from LKB, Sweden. Na[^{125}I] was supplied by Bhabha Atomic Research Centre, Bombay. Iodobead was obtained from Pierce Chemical Company, Rockford, Illinois.

Isolation of Spermatozoa

Spermatozoa were collected from fresh goat cauda epididymides by the procedure described before [2]. Highly motile spermatozoa were isolated at room temperature (30°C ± 1) from the epididymides in a modified Ringer's solution (RPS medium: 119 mM NaCl–5 mM KCl–1 mM $CaCl_2$–1.2 mM $MgSO_4$–10 mM glucose–16.3 mM potassium phosphate, pH 6.9–penicillin, 50 units/ml). Sperm numbers in the samples were determined with a haemocytometer.

Estimation of Anti-Sticking Activity

Anti-sticking activity was measured by the procedure reported earlier [3]. An aliquot of the sperm preparation (approx. 0.5×10^6 cells) was incubated at room temperature (30°C ± 1) with or without a specified amount of anti-sticking activity in a total volume of 0.5 ml of RPS medium. An aliquot of the cell suspension was then injected into the haemocytometer and the initial cell number was counted (C_T) under a phase contrast microscope. After 5 min of incubation, the counting chamber was flushed with an excess of RPS medium to remove the cells that were in suspension. The cells that were left in the chamber after flushing were then counted and these values (C_S) represent the cells that adhered firmly to the glass surface of the haemocytometer. The percentage of cells that did not adhere to the glass surface of the haemocytometer chamber was calculated as $[C_T - C_S/C_T] \times 100$. A unit of activity of ASF was defined as the amount of the factor that prevents sticking of 10% of the cells under the standard assay condition. Systems lacking exogenous anti-sticking activity served as the blank in all assays. The data shown are the means of duplicate determinations differing less than 15% and are representative of 3–4 experiments.

Purification of ASF-II

Freshly collected sperm preparations were centrifuged at 800*g* for 10 min and the resulting supernates were centrifuged again at 18,000*g* to obtain cell-free EP [3].

EP was stored at −10°C with PMSF (1 mM). ASF-II was isolated from the frozen samples of EP that were thawed and pooled.

Step 1: Concanavalin A–agarose affinity chromatography. EP was dialysed against 20 mM Tris-HCl, pH 7.2–1 mM $CaCl_2$–1 mM $MnCl_2$–0.6 NaCl (Buffer 1) and the dialysed material was subjected to affinity chromatography on a concanavalin A(Con A)–agarose column (1.8 × 15 cm) as described earlier [3]. The anti-sticking activity (ASF-I, II) that was immobilized on the column was eluted first with 150 ml of Buffer 1 containing 30 mg/ml of α-methyl-D-mannoside and then with 90 ml of 50 mM glycine-HCl, pH 3.0–1M NaCl–15 mg/ml of α-methyl-D-mannoside. The Con A eluate was neutralized, concentrated by filtration through Amicon PM-30 membrane, and then dialysed against 10 mM Tris-HCl, pH 9.0.

Step 2: DEAE-cellulose ion-exchange chromatography. The dialysed ASF preparation was subjected to a DEAE-cellulose chromatography for the resolution of ASF-I and II by the procedure reported earlier [3]. ASF-I bound to the resin was removed by eluting the DEAE-cellulose column (1.0 × 15 cm) with 30 ml of 0.1M Tris-HCl, pH 9.0. Undesirable proteins were removed by washing the column successively with 25 ml each of Tris-HCl pH 9.0 having concentrations of 0.2M, 0.3M, 0.5M, and 0.7M. ASF-II was finally eluted from the column with 25 ml of 1M Tris-HCl, pH 9.0–1M NaCl. Fractions containing ASF-II activity were concentrated by ultrafiltration through Amicon PM-30 membrane and then equilibrated with 5 mM potassium phosphate buffer (K-phosphate) pH 7.0.

Step 3: Alumina gel adsorption. Cγ-alumina gel (5 ml, 250 mg solid) was washed first with distilled water and then with 5 mM K-phosphate, pH 7.0, by centrifugation at 500*g* for 10 min. The preparation of ASF-II was mixed with the above gel and left in ice with constant stirring for 30 min. The mixture was then centrifuged at 500*g* for 10 min. The supernatant (unadsorbed fraction) was discarded and the gel was washed twice by centrifugation with 0.125M K-phosphate, pH 7.0. ASF-II was then eluted with 9 ml of 0.25M K-phosphate, pH 7.0. This active fraction was dialysed against 5 mM K-phosphate, pH 7.0 and readsorbed on the fresh alumina gel, prepared as above. ASF-II was eluted again with 9 ml of 0.25M K-phosphate, pH 7.0, concentrated by Amicon ultrafiltration using PM-30 membrane, and finally dialysed against 5 mM K-phosphate buffer, pH 7.0.

Step 4: Isoelectric focussing. ASF-II was further purified by isoelectric focussing in gel tubes (0.6 × 11 cm) using a discontinuous sucrose density gradient containing 2% Ampholine, pH range 5–8. A 10% polyacrylamide gel base (0.6 × 1.0 cm) containing 2% Ampholine was prepared at the bottom of the gel tubes to support the sucrose gradient. The polyacrylamide gel (without SDS) was prepared according to Laemmli [4]. ASF-II (from step 3) was added to 22% and 15% sucrose. Sucrose gradient was formed by layering successively 1 ml each of 22%, 15% and 10% sucrose on top of the polyacrylamide gel (personal communication from Prof. S. Bose, University of Notre Dame, South Bend, Indiana). Approx. 200 μg protein was loaded in each tube.

Isofocussing was carried out at 6°C initially for 4 hours at 200 V and then for 2 hours at 400 V using 0.1N NaOH and 0.1N acetic acid as the cathode and anode solutions, respectively. After the run, the gel was punctured with a needle and fractions of 2 drops each were collected. The fractions were assayed for ASF activity and the active fractions were pooled and dialysed against Buffer 1. To remove

Ampholine from the factor, the pooled fraction was passed through a small Con A–agarose column (0.5 × 3 cm) and ASF activity was eluted with 5 ml Buffer 1 containing 30 mg/ml of α-methyl-D-mannoside. This fraction was dialysed against 5 mM K-phosphate, pH 7.0 and then concentrated by dialysis against 30% glycerol–1 mM PMSF–5 mM K-phosphate, pH 7.0 and preserved in the same buffer at −10°C.

Determination of Sedimentation Constant

Sedimentation constant of ASF-II was estimated by sucrose density gradient fractionation [3]. For these studies, ASF-II was radioiodinated according to Marwell [5] with some modifications [3]. 0.1 ml of [^{125}I] ASF-II (approx. 25 ng protein; 28,000 cpm) containing 2 mg of ovalbumin as the marker protein was layered on top of 4.6 ml of a linear sucrose gradient (5–22%). The tubes were then centrifuged at 200,000*g* for 10 hours at 5°C in a Beckman SW 50.1 rotor. After the run, the bottom of the tube was punctured with a needle and 10-drop fractions (about 0.15 ml) were collected. The fractions were counted for radioactivity in a gamma-counter to localize the peak of ASF-II. The peak of the marker protein was determined by estimating the protein contents of the fractions.

Protein Estimation

Unless otherwise specified the samples were analyzed for their protein contents according to Lowry et al. [6] by using bovine serum albumin as standard. Protein concentrations of the samples containing glycerol were estimated by the method of Bensadoun and Weinstein [7].

RESULTS

Purification of ASF-II

ASF-II is a glycoprotein as it binds with high affinity to Con A (Table I). ASF-II showed markedly greater affinity than ASF-I for binding to DEAE-cellulose and based on this characteristic it has been possible to separate ASF-II from ASF-I: the major anti-sticking factor. ASF-II is adsorbed strongly by the alumina gel and most of the unwanted proteins could be removed by washing the gel with 125 mM K-phosphate, pH 7.0. This step alone resulted in a nearly 30 times increase in the purity of the factor. The factor was purified further by isoelectric focussing when only one peak of ASF activity having isoelectric point of 8.0 was obtained. By these steps ASF-II was purified nearly 1,700-fold (Table I). It showed a single protein band under non-

TABLE I. Purification of ASF-II From Goat Epididymal Plasma

Steps	Fractions	Total activity (units × 10^{-4})	Total protein (mg)	Specific activity (units/mg protein × 10^{-2})	Purification (-fold)
	Epididymal plasma	32.3	4,630	0.7	1
Step 1.	Con A-agarose chromatography	46.8	399	11.7	17
Step 2.	DEAE-cellulose chromatography: ASF-II	10.5	32	32.3	47
Step 3.	C_γ-alumina gel adsorption: ASF-II	9.3	0.93	996.7	1,445
Step 4.	Isoelectric focussing: ASF-II	9.2	0.78	1176.9	1,705

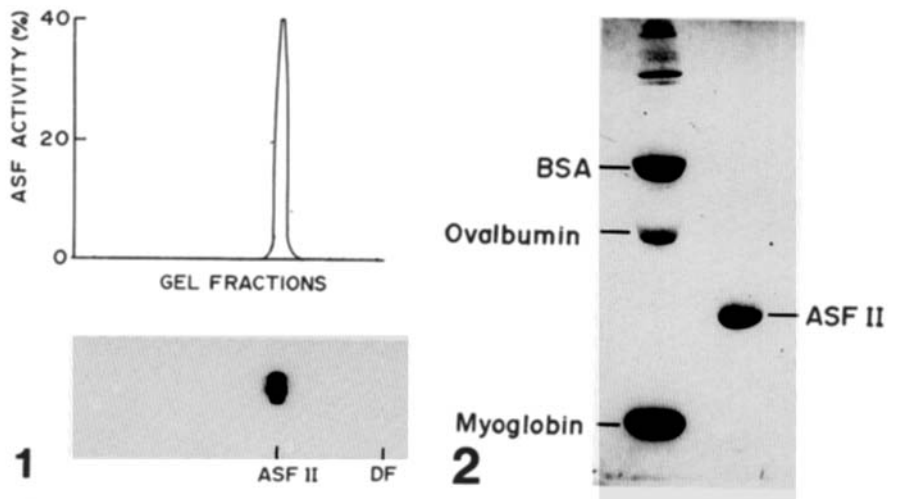

Fig. 1. Polyacrylamide gel electrophoretic pattern of ASF-II under the non-denaturing conditions (without SDS) according to Laemmli [4]. After electrophoresis one gel was stained with Coomassie blue for the detection of protein bands and another gel was sectioned with a Gilson automatic gel slicer for the detection of ASF activity in the gel, as described before [3].

Fig. 2. SDS-polyacrylamide gel electrophoretic pattern of ASF-II. Electrophoresis was performed by the method of Laemmli [4] under reducing condition in presence of 2-mercaptoethanol. **Right lane:** ASF-II; **left lane:** marker proteins—bovine serum albumin (BSA : 66 kDa), ovalbumin (45 kDa), and myoglobin (17 KDa).

denaturing (Fig. 1) and denaturing conditions (Fig. 2) of polyacrylamide gel electrophoresis and in the former case, the anti-sticking activity co-migrated with the protein band indicating apparent homogeneity of the factor. ASF-II showed a high degree of stability when preserved at −10°C in 5 mM K-phosphate, pH 7.0–1 mM PMSF–40% glycerol for about a month.

Physical Properties of ASF-II

The molecular weight of the purified ASF-II estimated by Sephadex G-100 gel filtration was found to be 36,000 (Fig. 3). Only one peak of anti-sticking activity was detected by the gel filtration of ASF-II. On SDS-gel electrophoresis ASF-II showed a single protein band with molecular weight of 35,000 (Fig. 2). Sucrose gradient ultracentrifugation studies showed that ASF-II has sedimentation coefficient of 2.4S (Fig. 4). The Stoke's radius of ASF-II calculated according to Ackers [9] was found to be 22.0 Å The frictional ratio of ASF-II estimated according to Siegel and Monty [10] was 1.0 indicating that the protein is spherical.

Dose Course of ASF

The activity of ASF-II increased linearly up to approx. 3 units of the factor (5.5 ng/ml or approx. 0.15 nM ASF-II) (data not shown). The inhibitory effect of the factor was nearly maximal (60%) at 30 ng/ml (approx. 1 nM) concentration of ASF-II. Previous studies demonstrated that purified ASF-I of EP at saturating concentration as well inhibited adhesion of 50–60% of sperm to glass [3]. There was no appreciable

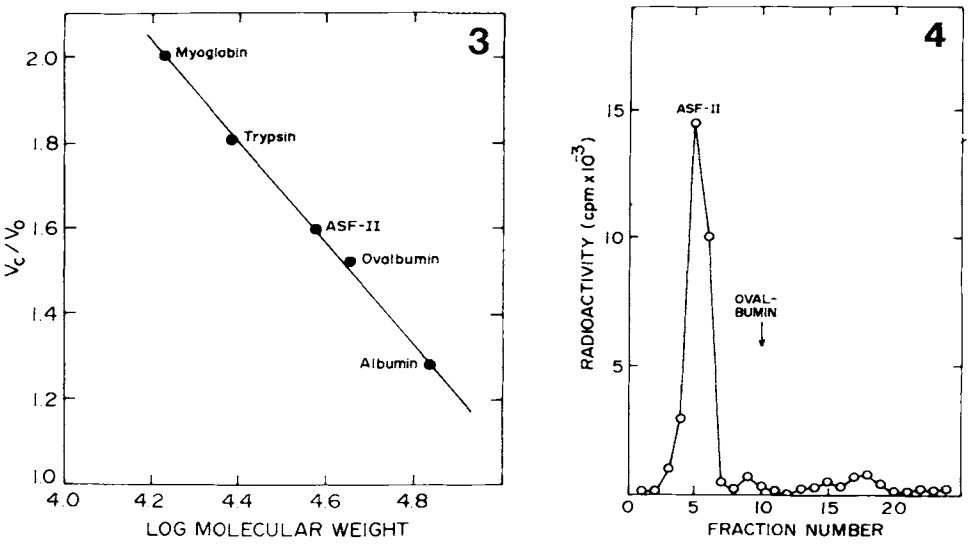

Fig. 3. Estimation of molecular mass of ASF-II by gel filtration on a column of Sephadex G-100 according to Whitaker [8] using bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsin (23 kDa), and myoglobin (17 kDa) as marker proteins.

Fig. 4. Sucrose density gradient profile of ^{125}I-labelled ASF-II. The fractionation was carried out by the procedure described in the Materials and Methods section. Ovalbumin (3.6S) was used as the marker protein.

further increase in the inhibition of sperm adhesion to glass when both the factors were added together at saturating concentrations (1.25 nM each) (data not shown).

Heat Stability

ASF-II is largely stable to heat treatment at 40°, 60°, and 80°C for 5 min. However, heating at 100°C for 5 min caused significant decrease (approx. 30%) in its activity (Table II).

Action of Glycosidases on ASF

Since the anti-sticking factor is a glycoprotein, it was treated with various glycosidases to investigate whether sugar residues are essential for its anti-sticking activity (Table III). Treatments with L-fucosidases (0.25 unit/ml) or α-mannosidase (17 units/ml) inhibited completely the activity of the factor. Neuraminidase (20 units/ml) inhibited partially the activity of ASF-II. β-galactosidase (200 units/ml) did not have any effect on the ASF-II activity. The data show that the sugar side-chains of the factor are essential for its anti-sticking activity.

Coating of Glass Surface

It is possible that ASF may show its anti-sticking activity by coating the haemocytometer counting chamber. To test this possibility, the counting chamber was separately incubated first with saturating concentration (100 ng/ml) of ASF-II in RPS medium for 5 min at room temperature and then flushed with excess of RPS medium to remove the free factors. This pretreatment of the chamber had no effect on cells

TABLE II. Effect of Heat Treatment on the Activity of ASF-II*

Heat treatment	Anti-sticking activity (units)
Nil	3.2
60°C	3.0
80°C	3.1
100°C	2.2

*ASF-II (approx. 100 ng/ml) was heated at 60°C, 80°C, and 100°C for 5 min, cooled to room temperature, and then assayed for anti-sticking activity under the standard assay conditions. The concentration of the heat-treated factors used in the assays was approx. 5 ng/ml.

TABLE III. Effect of Glycosidases on the Activity of ASF-II*

Systems	ASF-II activity (units)
ASF-II	3.2
ASF-II + L-fucosidase (0.25 units/ml)	0.0
ASF-II + α-mannosidase (17 units/ml)	0.0
ASF-II + Neuraminidase (20 units/ml)	2.3
ASF-II + β-galactosidase (200 units/ml)	2.7
ASF-II + β-N-acetylglucosaminidase (1.6 units/ml)	2.8

*ASF-II (4 ng) was pretreated with specified concentrations of the glycosidases in a total volume of 50 μl of RPS medium at 37°C for 2 hours. After incubation the mixtures were heated at 80°C for 5 min to destroy the glycosidase activities and the samples were then assayed for anti-sticking activity under standard assay conditions. The glycosidases had no anti-sticking activity of their own.

sticking to glass, indicating that the activity of the factor was not due to coating of glass (data not shown).

Binding of ASF to Cells

ASF-II (50 ng/ml) when treated at room temperature for 10 min with an excess of washed spermatozoa (50×10^6/ml) in RPS medium was removed from the medium and the anti-sticking activity was found to be associated with the cells (data not shown). The results suggest that ASF-II binds to sperm surface proteins responsible for cell sticking to glass.

Effect of Metal Ions

Previous studies showed that bivalent metal ions may play a role in sperm adhesion to glass [2]. It is possible that ASF-II may show its anti-sticking activity by a chelating action. This, however, appears unlikely since $MgCl_2$ (1 mM) and/or $CaCl_2$ (1 mM) had no effect on the activity of ASF-II (data not shown).

Effect of ASF of Sperm Motility

Although it has been postulated that sperm adhesion to the glass chamber of a haemocytometer may pose as an artifact in motility assays [1,2], there has been no definite evidence to show that forward-motile cells really stick to glass. To investigate this possibility, vigorously forward-motile spermatozoa, separated specifically from the non-motile and weakly motile cells on the basis of their capacity to move upward

against gravity [11,12], were evaluated for their affinity to stick to glass in the absence of any anti-sticking factors. It has been observed that nearly 100% of the vigorously motile cells adhere to glass (data not shown), indicating that cell adhesion to glass will definitely give rise to artifacts in motility assays.

The effects of ASF-II on sperm motility were determined by using the spectrophotometric assay of motility [13], a method which is quantitative and largely rules out the possibility of cell-sticking artifacts in motility assays (Fig. 5). The initial slopes of the curves represent the index of the velocity of the population of cells showing the fastest motility. The sperm preparations had approx. 30% of spermatozoa with vigorous motility as shown by the spectrophotometric assay. ASF-II (approx. 1.25 nM) had little effect on the initial slopes as well as on the plateau values of the "motility" curves. Nearly identical results were also obtained with purified ASF-I. The data show that ASF-I and II have no appreciable effect on the velocity of the forward-motile cells and they were also ineffective in causing induction of forward progression in non-motile/weakly motile cells.

Studies on the Possibility of the Origin of ASF-II From ASF-I

ASF-I, having a molecular mass of 47 kDa [3], is a slightly larger-sized protein than ASF-II (Fig. 3). Investigations were therefore carried out to determine whether ASF-II is derived from ASF-I as a result of its enzymatic (proteases and/or glycosidases) modification during the purification procedure. Each of the purified anti-sticking factors showed the presence of a single peak of ASF activity when subjected to isoelectric focussing, Sephadex gel filtration, sucrose-gradient centrifugation, and

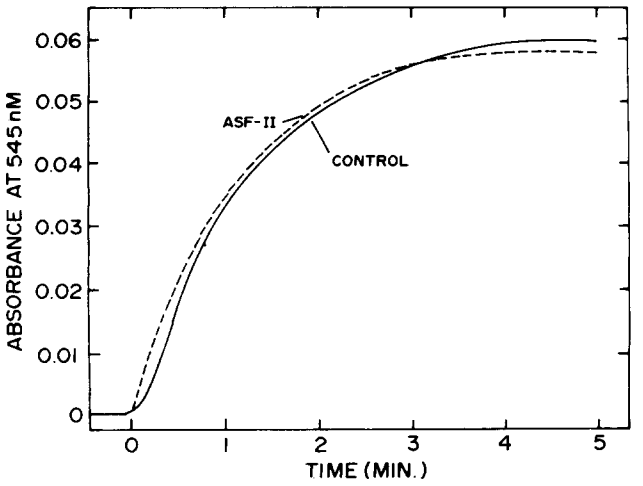

Fig. 5. Effect of ASF-II (100 ng/ml) on sperm forward motility estimated spectrophotometrically [13]. The method consists of layering of 50 μl of freshly extracted goat cauda-epididymal spermatozoa (10×10^6 cells) suspended in RPS medium containing 2% Ficoll-400 at the bottom of a standard optical cuvette containing 1.3 ml RPS medium which was sufficient to cover the entire width of the light beam. Vigorously motile spermatozoa, that moved upwards in the light beam, were registered continuously as an increase of absorbance at 545 nm with a Gilford spectrophotometer equipped with a recorder. After reaching the maximal absorbance (A_{Eq}), the contents of the cuvette were mixed and the absorbance for all the cells was noted (A_T). The percentage of cells that showed vigorous forward motility was calculated as $A_{Eq}/A_T \times 100$.

polyacrylamide gel electrophoresis, indicating that ASF-I and -II are not in equilibrium. To minimize the possibility of modification of ASF-I by proteases and/or glycosidases, EP was first extracted from epididymides in presence of PMSF (1 mM) the protease inhibitor and then subjected immediately (within 1 hour) to DEAE-cellulose chromatography (omitting Con A–agarose affinity step) for the resolution of ASF-I and -II. The profile of ASF-I and -II were nearly identical in the freshly extracted or stored EP, suggesting that ASF-II has not been derived from ASF-I.

DISCUSSION

A previous study from this laboratory has characterized ASF-I, the major anti-sticking factor of goat EP [3]. This study reports purification and characterization of ASF-II: another anti-sticking factor from EP. ASF-II is a glycoprotein as indicated by its binding to Con A and it is sensitive to the action of some glycosidases (Tables I, III). It is a specific anti-sticking protein since, as demonstrated earlier, several purified proteins showed little or markedly lower anti-sticking potency [3]. ASF-II closely resembles ASF-I in respect to several biochemical characteristics. For example, both factors are heat-stable glyco-proteins and both the protein and sugar side-chains of these factors are essential for their ASF activity. Despite close similarities of certain properties of ASF-I and -II, ASF-II is clearly different from ASF-I, as evidenced by its lower molecular mass and sedimentation constant (Figs. 3, 4) and markedly higher affinity for binding to DEAE-cellulose [3]. The finding that ASF-I rather than ASF-II is sensitive to treatment with β-N-acetyl glucosaminidase [3] is consistent with this view. This contention is strengthened further by the observations that ASF-I and -II are not in equilibrium and that freshly extracted EP and stored EP possess nearly identical ratios of these factors.

As indicated above, sperm adhesion to a glass surface may serve as a serious artifact in sperm motility estimations [1,2] since haemocytometer chambers are widely used for motility assays [14]. The earlier investigators, while reporting the occurrence of various motility-promoting and maintenance factors in the male reproductive fluids and blood serum [15–21], have not taken into consideration the possibility of cell-sticking artifacts in the motility assays. It is thus possible that the previous studies on motility estimations with special reference to those on the occurrence of various motility factors may be complicated by artifacts due to sperm adhesion to glass. The observation that the anti-sticking factors that are present in the natural environment of sperm have no effect on sperm motility (Fig. 5) indicates that ASFs are ideally suitable for improving existing motility assays by eliminating the possibility of cell-sticking artifacts. This view is strengthened by the finding that EP derived from goat and ram showed ASF activity towards homologous and heterologous spermatozoa (unpublished results).

As shown earlier, EP possesses anti-sticking activity that prevents adhesion of 100% of the spermatozoa to the glass surface [2]. The observation that at saturating concentration ASF-I or -II alone or together inhibited adhesion of only 50–60% spermatozoa shows that these cells are sensitive to the actions of both ASF-I and -II. It appears possible that both ASF-I and -II may use the same cell-surface attachment site (receptor) of these sperm population (50–60%). The rest of the sperm population (40–50%) is insensitive to the action of ASF-I and -II presumably due to absence/inadequate amount of receptor sites on these cells. The data imply that in addition to

ASF-I and -II, EP also possesses other ASFs which were inactivated or not recovered during the purification steps and these unidentified ASFs may perhaps be responsible for actions on ASF-I- and -II-insensitive spermatozoa.

The physiological significance of the anti-sticking factors is largely unknown. The observed sperm-glass adhesion is an example of cell-substratum interaction which closely resembles that of cell-cell adhesion [22,23]. As speculated earlier [3], the anti-sticking factors may as well inhibit sperm-sperm adhesion and the adhesion of sperm to the inner surfaces of the male and female reproductive tracts. The ASFs may thus serve an important function by permitting sperm to remain in a free state essential for maintaining their motility and fertility potential.

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REFERENCES

1. Stephens DT, Acott TS, Hoskins DD: Biol Reprod 25:945–949, 1981.
2. Roy N, Majumder GC, Chakrabarti CK: Andrologia 17:200–206, 1985.
3. Roy N, Majumder GC: Biochim Biophys Acta 991:114–122, 1989.
4. Laemmli UK: Nature 227:680–685, 1970.
5. Markwell MAK: Anal Biochem 125:427–432, 1982.
6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265–275, 1951.
7. Bensadoun A, Weinstein D: Anal Biochem 70:241–250, 1976.
8. Whitaker JR: Anal Chem 35:1950–1953, 1963.
9. Ackers GK: Biochemistry 3:723–730, 1964.
10. Siegal LM, Monty KJ: Biochim Biophys Acta 112:346–362, 1966.
11. Sokoloski JE, Blasco L, Storey BT, Wolf DP: Fertil Steril 28:1337–1341, 1977.
12. Barua M, Bhattacharyya U, Majumder GC: Biochem Int 10:733–741, 1985.
13. Majumder GC, Chakrabarti CK: J Reprod Fertil 70:235–241, 1984.
14. Amelar RD, Dubin L, Schoenfeld C: Fertil Steril 34:197–215, 1980.
15. Morita Z, Chang MC: J Reprod Fertil 24:247–254, 1971
16. Morton B, Chang TSK: J Reprod Fertil 35:255–263, 1973.
17. Gaur RD, Talwar GP: Int J Fertil 20:133–136, 1975.
18. Muller B, Kirchner C: J Reprod Fertil 54:167–172, 1978.
19. Morton BE, Fraser CF, Sagadraca R: Fertil Steril 32:107–114, 1979.
20. Brown DV, Senger PL: Biol Reprod 23:271–275, 1980.
21. Chaudhury DP, Majumder GC: Andrologia 15:495–497, 1983.
22. Hughes RC, Pena SDJ, Vischer P: In Curtis ASG, Pitts JD (eds): "Cell Adhesion and Motility," The 3rd Symposium of the British Society for Cell Biology. Cambridge, U.K: Cambridge University Press, 1980, pp 329–356.
23. Garrod DR: J Cell Sci [Suppl] 4:221–237, 1986.