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Purification and Characterization of a Motility Initiating Protein From Caprine Epididymal Plasma

BIJAY SHANKAR JAISWAL,[1] KAUSHIK DAS,[1] SUDIPTA SAHA,[1] SANDHYA REKHA DUNGDUNG,[1] AND GOPAL C. MAJUMDER[1,2]*

[1]*Indian Institute of Chemical Biology, Kolkata, India*

[2]*Centre for Rural and Cryogenic Technologies, Jadavpur University, Kolkata, India*

Numerous reports have appeared on the occurrence of undefined protein factors in male reproductive fluids that promote motility of mature sperm and initiate forward motility in the immature (immotile) caput-epididymal sperm. This study reports for the first time purification to apparent homogeneity of a motility initiating protein (MIP) from epididymal plasma and its characterization using the caprine sperm model. It is a 125 kDa (approximately) dimeric protein made up of two subunits: 70 and 54 kDa. MIP is an acidic protein with an isoelectric point of 4.75. The motility protein at 30 μg/ml (240 nM) level showed nearly maximal motility-promoting activity. MIP is heat stable and it is maximally active at pH 8. It is a glycoprotein that binds with high affinity to concanavalin A and it contains mannose, galactose, and *N*-acetyl glucosamine approximately in the ratios of 6:1:6. It is sensitive to the actions of α-mannosidase and β-*N*-acetylglucoseaminidase thereby demonstrating that the sugar side chain of the glycoprotein is essential for its biological activity. Epididymal plasma is its richest source. It is also capable of enhancing forward motility of mature cauda-sperm. Its antibody markedly inhibits sperm motility. MIP antibody is highly immunospecific and it recognizes both the subunits. MIP causes significant increase of the intrasperm level of cyclic AMP. MIP: the physiological motility-activating protein has potential for use as a contraceptive vaccine and for solving some of the problems of human infertility and animal breeding.

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Mammalian testicular spermatozoa are immotile and infertile. During transit through epididymis these cells acquire forward motility, which is essential for their ability to fertilize the eggs. The biochemical mechanism of sperm forward initiation in epididymis and its regulation is largely unknown. As reviewed earlier (Mandal et al., 1989; Majumder and Mandal, 1992; Majumder et al., 2001), early workers have reported the occurrence of various types of uncharacterized motility regulating protein factors in male reproductive fluids/reproductive organs (Morton and Chang, 1973; Gaur and Talwar, 1975; Sheth et al., 1981; Tso and Lee, 2008). Hoskins and his associates (Acott and Hoskins, 1978; Hoskins et al., 1978) have developed a novel in vitro model that permitted initiation of forward motility in the immature (immotile) sperm derived from bovine caput-epididymis when incubated in presence of epididymal plasma/seminal plasma and theophylline. Subsequently several investigators using multiple mammalian species have demonstrated that optimal in vitro initiation of forward motility in the immature caput-sperm requires four exogenous parameters: theophylline, epididymal plasma, bicarbonate, and alkaline pH (Hoskins et al., 1978; Kann and Serres, 1980; Cornwall et al., 1986; Jaiswal and Majumder, 1996, 1998). Bicarbonate works by elevating the intrasperm level of cyclic AMP (Pinto et al., 1984; Tajima et al., 1987; Rojas et al., 1992; Jaiswal and Majumder, 1998) whereas theophylline enhances level of sperm cyclic AMP by inhibiting cyclic phosphodiesterase (Hoskins et al., 1978; Jaiswal and Majumder, 1998). These findings show that intrasperm cyclic AMP enhances sperm motility. Evidence has been provided to support the view that epididymal plasma possesses an unknown protein factor that works in concert with theophylline for the induction of forward motility in the immotile sperm (Acott and Hoskins, 1978; Hoskins et al., 1978; Pinto et al., 1984; Jaiswal and Majumder, 1998). Hoskins and his associates (Acott and Hoskins, 1978; Hoskins et al., 1978) have partially purified the active principle from bovine seminal plasma and epididymal plasma and designated it as forward motility protein (FMP). The isolated preparation of FMP contained several proteins as shown by SDS–gel electrophoresis and the extent of purity of the motility protein is not known. Ding et al. (2007) have provided evidence to show that human seminal plasma contains sperm forward motility-related proteins that may have similarity with the precursors of alpha-1-antitrypsin and zinc-alpha-2-glycoprotein. It is thus clear that although many reports have appeared on the occurrence of undefined motility regulating proteins in male reproductive fluids, the motility proteins have not been adequately purified and characterized. Present study reports for the first time purification to apparent homogeneity of a motility initiating protein (MIP) from

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Bijay Shankar Jaiswal's present address is Department of Molecular Biology, MS 413a, Genentech, Inc., 1DNA Way, South San Francisco, CA 94080.

Kaushik Das's present address is Department of Molecular Medicine, Bose Institute, Centenary Building, Kolkata 700054, India.

*Correspondence to: Gopal C. Majumder, AICTE Emititus Fellow, Centre for Rural and Cryogenic Technologies (CRCT), Jadavpur University, Kolkata 700032, India.
E-mail: majumdergc42@yahoo.co.in

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epididymal plasma using the caprine model and some of its biochemical and functional characteristics.

Materials and Methods

Ammonium-sulphate, DEAE-cellulose, CM-cellulose, Concanavalin A-Sepharose, polyethyleneglycol, methyl-α-D-mannopyranoside, α-fucosidase, glucosidase, β-*N*-acetylglucosaminidase, α-mannosidase, neuraminidase, β-galactosidase, glucose oxidase, Ficoll-400, phenylmethylsulfonylfluoride, orthophenylenediamine, 4-chloro-1-napthol, HRP-conjugated goat antirabbit IgG, gelatin, fetuin, calcium hydrogen phosphate gel, Cγ-alumina gel, bovine serum albumin (BSA), ovalbimin, imidazole, trypsin, SDS markers (MW-SWS-200), non-denatured molecular weight markers (MW-ND-500), gel filtration markers (MW-GF-200), ampholine, and broad PI-calibration Kit (3–10) were obtained from Sigma Chemical Company (St. Louis, MO). Poly buffer exchanger (PBE-94) and poly buffer 74 (PB-74) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Microtitre Plates were obtained from Gibco (Grand Island, New York). Antisticking factor (ASF) that inhibits non-specific sperm adhesion to the glass surface of hemocytometer was isolated from goat epididymal plasma as described earlier (Roy and Majumder, 1989).

Isolation of spermatozoa and epididymal plasma

Immature and mature spermatozoa were obtained from goat caput- and cauda-epididymides, respectively by the procedure as described earlier (Roy et al., 1985). Spermatozoa were extracted from epididymis by making few incisions in epididymis at room temperature (32 ± 1°C) with a modified Ringer's solution (RPS medium: 119 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, penicillin 50 U/ml, 16.3 mM potassium phosphate, pH 7.5). Unlike the cauda-sperm preparations, the caput cell preparation contained large amount of fat globules. A homogeneous population of caput-spermatozoa was obtained by discontinuous Ficoll-400 (Sigma) gradient centrifugation procedure (Halder et al., 1990). The method consists of layering the freshly prepared caput-sperm suspension on the top of Ficoll gradient of 2.0 ml each of 2%, 4%, 6%, and 8% Ficoll in RPS medium. The cells were then centrifuged at 100*g* for 8 min at room temperature (32 ± 1°C) in a swing bucket centrifuge. The cells that sedimented at the bottom of the 8% gradient were collected and dispersed in RPS medium. These cells were free from the contaminating blood cells and fat globules. Numbers of spermatozoa in the samples were determined with a hemocytometer. The sperm preparations were used immediately (within 30 min) for motility assays after appropriate dilution in the RPS medium. Epididymal plasma was prepared from goat cauda-epididymis by the procedure reported earlier (Roy et al., 1985). Epididymal plasma was dialyzed against RPS medium to remove all dialyzable constituents including bicarbonate. The dialyzed epididymal plasma was used for assay of MIP activity and for its purification.

Assay of MIP activity

The activity of MIP that causes induction of forward motility in immature caput-sperm was measured by the procedure described earlier (Jaiswal and Majumder, 1998) with some modification. ASF that inhibits non-specific sperm adhesion to the glass surface of hemocytometer was isolated from goat epididymal plasma as described earlier (Roy et al., 1985). Caput-spermatozoa (25×10^4 cells) were incubated with 30 mM theophylline, ASF (250 μg/ml) and in absence or presence of specified amount of epididymal plasma (as a source of MIP)/its fractions/purified MIP at room temperature (32 ± 1°C) for 10 min in 250 μl of RPS medium. A portion of the cell suspension (5 μl) was then injected into the hemocytometer. Immediately spermatozoa that showed well defined forward motility (excluding cells that moved in small or large circles) and total cell numbers were counted under a phase contrast microscope at 400× magnification. The percentage of forward motile cells was then calculated. System lacking MIP served as the blank in all assays. A unit of MIP activity was defined as the amount of the factor that induced forward motility in 10% of the immature cells under the standard assay conditions.

Purification of MIP

The epididymal plasma proteins were fractionated using 0–30%, 30–50%, and 50–70% saturation of ammonium sulphate. In each step, the mixture of proteins suspension and salt was centrifuged for 15 min at 18,000*g* and the sedimented protein pellet was dissolved in 10 mM potassium phosphate, pH 8.0, while the supernatant fluids were subjected to further saturation by addition of the solid salt. MIP activity was largely precipitated (about 90%) by 30–70% saturation of $(NH_4)_2SO_4$. The active fraction was dialyzed against 10 mM potassium phosphate buffer, pH 8.0.

The resulting ammonium sulfate fraction of MIP was applied at a constant flow rate of 1 ml/min to DEAE-anion exchange column (7.5 cm × 0.75 cm) on Waters HPLC system that was pre-equilibrated with 10 mM potassium phosphate, pH 8.0. The column was then eluted at a flow rate of 1 ml/min using the following regime: (1) a linear gradient of 10–200 mM potassium phosphate, pH 8.0, for a volume of 15 ml, (2) 200 mM potassium phosphate, pH 8.0, for a volume of 10 ml, (3) Another reverse linear gradient of 200–10 mM potassium phosphate, pH 8.0, for a volume of 2 ml, (4) 10 mM potassium phosphate, pH 8.0, for a volume of 20 ml. Protein elution was monitored at 280 nm and peaks were recorded manually and assayed for MIP activity after dialysis against RPS medium. The active MIP fractions were then pooled and concentrated by Amicon ultrafiltration with PM-30 membrane. Concanavalin A immobilized on Sepharose, is known to have high affinity for binding D-mannose and D-glucose residues of the glycorpoteins. ConA-Sepharose column (1 cm × 12 cm: bed volume 10 ml) was equilibrated with buffer I (20 mM Tris–HCl, pH 7.2, containing 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 1.0 mM $MnCl_2$). The above-mentioned MIP fraction was dialyzed extensively against buffer-I, prior to its loading on the ConA-Sepharose affinity column. As the sample passed through, the column was washed with 15 ml of buffer-I. The elute from the column represents the unretained fraction (fraction A). The column was washed further with 50 ml of the equilibrating buffer to remove the residual amount of unbound material. Finally, the glycoproteins that bound to the affinity column were eluted with 60 ml buffer-I containing 0.5 M α-methyl-D-mannopyranoside (fraction B). All the fractions were dialyzed extensively against RPS medium prior to assay of MIP activity. MIP was purified further by chromatofocusing on PBE 94 (Sluyterman and Wijdenes, 1978). A column of ion exchange resin, PBE 94 (0.7 cm × 10 cm), was equilibrated with 25 mM imidazole buffer, pH 7.4, and the sample was applied to it. After the sample passed through, the column was washed with the eluting buffer. The MIP activity was eluted with polybuffer 74 (Pharmacia Fine Chemicals), pH 4.0, (1:8 dilution) in 1 ml fractions. The elution was monitored at 280 nm absorbance and pH of each fraction was measured. MIP activity (indicated by arrow in Fig. S1) were pooled, concentrated and finally dialyzed against 10 mM potassium phosphate, pH 6.9, before activity measurement. Pooled and concentrated MIP sample after chromatofocusing was subjected to Cγ-alumina gel adsorption. Ten milliliters alumina gel suspension (2.5 g solid) was centrifuged at 500*g* for 10 min at 4°C. The pellet was then washed twice successively with 10 mM potassium phosphate, pH 6.0. The MIP preparation was mixed with the above gel and left in ice with constant stirring for 45 min. The mixture was then centrifuged at 500*g* for 10 min and the resulting supernatant fraction was discarded. Bound proteins were then eluted successively with 20 ml each of 0.1, 0.25, 0.5, and finally with 1 M potassium phosphate, pH 6.9, containing 1 M NaCl. Before activity

measurement, all the fractions were dialyzed against RPS medium and concentrated. Major portion of MIP activity was eluted with 0.5 M potassium phosphate, pH 7.0. The MIP preparation was then loaded onto a Sephacryl S-200 gel filtration column (0.9 cm × 50 cm) previously equilibrated with RPS medium. The fractions (1.0 ml/tube) were collected in a LKB fraction collector. The active fractions were pooled, concentrated and stored at −20°C.

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions

To check the homogeneity, purified MIP was analyzed by PAGE under non-denaturing conditions (Laemmli 1970). MIP was dialyzed in sample buffer and subjected to 6% polyacrylamide gel electrophoresis. After electrophoresis the gel was stained with silver nitrate (Wray et al., 1981). MIP was also subjected to polyacrylamide gel electrophoresis using different percentages of the gel 5%, 6%, 7%, and 8%. The known protein markers (Sigma) such as urease (272 kDa), BSA (Dimer 132 kDa and monomer 66 kDa), chicken egg albumin (45 kDa), and carbonic anhydrase (29 kDa) were run simultaneously. The molecular weight of each protein was determined first by plotting 100 log ($R_f \times 100$) against the percentage of gel for measurement of retardation coefficient (K_R) of each protein and then by plotting K_R against log MW of known markers (Davis 1964; Ferguson 1964; Bryan 1977). The molecular weight of MIP was determined from the standard curve.

Determination of molecular weight and subunit composition

The native molecular weight of MIP was estimated using a column of Sephacryl S-200 (0.9 cm × 50 cm) previously equilibrated with RPS medium. Fractions (1 ml) were collected and protein was monitored by absorbance at 280 nm. The column was calibrated with known molecular weight marker proteins: such as cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase b (97.6 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). The molecular weight of MIP was also determined by analytical HPLC. The MIP was passed through a gel filtration column of HPLC. For determination of molecular weight the column was calibrated with some known molecular weight markers such as apoferritin (440 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). To determine the subunit composition of MIP, the factor was subjected to SDS–PAGE according to Laemmli (1970). Markers used for determination of molecular weight of the MIP subunits, were β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20 kDa).

Determination of isoelectric point

To determine the isoelectric point of MIP, analytical isoelectric focusing was carried out according to, Låås et al. (1980). A set of Pharmacia standard proteins (with known PI) was run side by side for determining the PI of MIP. The protein bands were detected by staining with 0.1% Coomassie Blue G-250 in an aqueous solution containing 25% methanol 5% acetic acid (w/v). Isoelectric point of MIP was further confirmed by chromatofocusing using PBE 94 (Sluyterman and Wijdenes, 1978). A column of ion-exchange resin, PBE 94 (0.70 cm × 10 cm) was equilibrated with 0.25 M imidazole–HCl, pH 7.4. The sample was dialyzed against the same buffer prior to its application to the chromatofocusing column. After passage of the sample, MIP was eluted with polybuffer 74-HCl, pH 4. The volume in each fraction was 1.2 ml. Protein was monitored by following absorbance at 280 nm. The pH values of the fractions were determined with a pH meter. The PI was determined by assaying the MIP activity of the fractions.

Detection of sugar residues

For detection of sugar residues, the purified MIP was hydrolyzed with 2 M-trifluoroacetic acid (Albersheim et al., 1967) in a sealed tube at 120°C for 1½ h. The acid was removed under vacuum. Traces of acid were removed by co-distillation with water. The hydrolysate was then reduced with sodium borohydride and converted into alditol acetate (Sloneker, 1972). The neutral and amino sugars were detected as their acetates by gas liquid chromatography using a Hewlett Packard 5890 series II gas chromatograph with a flame ionization detector. Resolutions were performed on glass columns (1.83 mm × 2 mm) containing (a) 3% of sp 2340 on supelcoport (100–200 mesh) at 200°C (for alditol acetates of neutral sugars); (b) 3% of poly A-103 on Gas Chrom Q (80–100 mesh) at 200°C (for alditol acetates of amino sugars). A recording integrator HP 3396A (Hewlett Packard, Palo Alto) was used to determine peak areas.

Raising the polyclonal antibody against the purified MIP

Antiserum against the purified MIP was raised in rabbit by four successive injections (Ouchterlony, 1958). the immunoglobulin of the immune serum was precipitated twice with 50% ammonium sulfate. The final precipitate was dissolved in 0.2 M PBS, pH 8.0, and excess ammonium sulphate was removed by dialysis against the same buffer. The immunoglobulin fraction obtained after the salt fractionation was isolated by passing through DEAE-cellulose column. The purified IgG was collected in 0.2 M PBS, pH 8.0, and dialyzed against the same buffer.

ELISA

Standard ELISA curves were run with increasing concentrations (75 to 375 ng) of highly purified MIP. We added 60 μg protein of tissues and 8 μg protein of reproductive fluid in each well of the microtitre plate and incubated the plate overnight at 4°C. After incubation each well was washed with PBS and then non-specific adhesion was blocked with 3% BSA in PBS and further incubation was carried out at 37°C for 1 h. After this incubation 1st antibody (antibody of MIP) was added to each well (dilution 1:1,000) diluted with 1% BSA in PBS. Incubation and further washing of the wells were done as previously followed by addition of HRP-conjugated goat antirabbit IgG (2nd antibody, 1:2,000 dilution in PBS containing 1% BSA). After the addition of 2nd antibody the plate was incubated at 37°C for 60 min and then washed thoroughly with PBS. Finally color reaction was developed using 3 mM orthophenyl diamine (OPD) (Sigma) in 24 mM citric acid, 50 mM dibasic sodium phosphate, containing 0.04% of a 30% H_2O_2 stock (pH 5) (Wisdom, 1976) as a substrate. Development of color was stopped after 20 min with 4(N) H_2SO_4 and optical density was measured at 492 nm using an ELISA reader.

Western blotting

For determination of immunospecificity, MIP antibody was evaluated by Western blot procedure. Epididymal plasma was run on PAGE and transferred to nitrocellulose paper by Bio-Rad transblot apparatus. The immunoblot was carried out according to the procedure of Towbin et al., 1979. Non-specific binding sites were blocked with 3% skimmed milk in TBS (10 mM Tris–HCl, pH 7.5, containing 0.9% NaCl) for 1 h at 37°C. The nitrocellulose paper was then incubated with 1st antibody (MIP antibody), diluted at 1:2,000 in TBS containing 1% skimmed milk, overnight at 4°C. Then the blot was washed with TBS containing 0.01% Tween-20 and after that with only TBS. After washing the blot was incubated in alkaline phosphatase-conjugated goat antirabbit IgG (2nd antibody in TBS–1% skimmed milk at 1:1,000 dilution) for 1 h at room temperature (32 ± 1°C). After further washing, immunoreactive band was visualized using NBT-BCIP as a chromogenic substrate for alkaline phosphatase.

Other methods

For performing Ouchterlony double diffusion experiment a gel slide was prepared with 1% agarose in 0.9% NaCl containing 0.1% sodium azide. The central well contained the purified MIP. The surrounding wells contained different lectins such as concanavalin A (ConA), wheat germ agglutinin (WGA), Ricinus communis agglutinin (RCA2). The slide was incubated for 24–48 h at 4°C in a humid atmosphere.

The protein contents of the samples were estimated according to (Lowry et al., 1951) using BSA as standard. All experiments were repeated at least three times. The data are presented as the mean ± standard error of the mean (SEM). Significance of difference between treated (epididymal plasma/MIP/MIP antibody) and control (absence of MIP or epididymal plasma/normal rabbit serum/PBS–BSA) was calculated by paired as well as unpaired Student's *t*-test.

Results

Purification of MIP

Summary of the purification of MIP has been shown in Table 1. When 30–70% ammonium sulphate fractionation was performed with crude epididymal plasma, the recovery of MIP activity was 260%. The biochemical basis of this increase in recovery is not clear. It may be due to removal of some inhibitors from epididymal plasma. By this step MIP was purified to eightfold (Table 1). MIP binds to DEAE-HPLC resin and was eluted with the linear gradient of potassium phosphate (10–200 mM) buffer, pH 8.0, (data not shown). MIP was eluted at phosphate concentration of 165–185 mM as the major active peak having retention time of 18 min. MIP activity binds to the Concanavalin A-Sepharose affinity matrix and the bound MIP was eluted with methyl α-D-mannopyranoside (Fig. 1) implicating thereby that MIP is a glycoprotein. When the active MIP from ConA-Sepharose was subjected to chromatofocusing, one major and two small protein peaks were obtained (Fig. S1). Assay of MIP activity showed that MIP activity was present in the major protein peak. The isoelectric point of MIP was found to be 4.8–5.1. Nearly 90% of the MIP activity was adsorbed by alumina gel and the activity could be recovered from the gel by elution with 0.5 (M) potassium phosphate buffer, pH 6.9. The MIP preparation was further purified by Sephacryl S-200 gel filtration (data not shown). The isolated MIP was ~500-fold purified.

Physical properties of MIP

Purity of MIP was checked by native gel electrophoresis. The electrophoretogram showed a single protein band (Fig. 2A). One gel was stained with silver nitrate and from another gel MIP activity was eluted. MIP activity co-migrated with the protein band (Fig. 2B). The purity of MIP was further supported by different percentages of native polyacrylamide gel electrophoresis (Fig. S2). MIP showed a single protein band under all electrophoretic conditions. A single protein peak was also obtained by isoelectric focusing (Fig. 3) and HPLC (Fig. 4). These data establish apparent homogeneity of the purified MIP. The molecular weight of MIP estimated by Sephacryl S-200 gel filtration was 125 kDa (data not shown). A single sharp peak of activity was obtained when MIP was subjected to Sephacryl S-200 gel filtration. The molecular weight of MIP was ~120 kDa when analyzed by HPLC (Fig. 4). By both the techniques a single symmetric activity peak was obtained in the MIP preparation. The molecular weight of MIP estimated by the method based on different percentages of polyacrylamide gel was 118 kDa (Fig. S2). Subunit composition of MIP was determined by SDS–PAGE (Fig. 5). Two protein bands having molecular weight of 70 and 54 kDa were observed in the gel electrophoretogram (Fig. 5). By this method MIP has molecular weight of 124 kDa, indicating thereby that MIP is a dimeric protein. the isoelectric point of MIP was around 4.75 (Fig. 3). From another polyacrylamide gel, MIP was eluted and its activity was measured. The MIP activity co-migrated with the protein band (data not shown). The PI of MIP was also supported by chromatofocusing data (Fig. S3).

Stoke's radius

The Stoke's radius: a, of MIP was calculated according to the formula, $M = \pi 4\, Na^3/3V$ (Ackers, 1964) (where a is the Stoke's radius (cm), M is molecular weight, V is partial specific volume (cm^3/g; assumed to be 0.75 cm^3/g); $\pi = 3.14$ and N is Avogadro's number (6.023×10^{23}/mol). Stoke's radius of MIP was found to be 33.38 Å.

Biochemical properties of MIP

Freshly extracted goat caput-sperm (immature) preparations do not show forward motility when analyzed in absence of exogenous MIP. Addition of MIP induces forward motility to a significant population of spermatozoa (Fig. 6). The numbers of forward motile cells increased markedly with the increase in the concentration of MIP. The factor showed maximal activity at concentration as low as 30 μg/ml when it induces forward motility in nearly 22% of the immature spermatozoa. The motility-promoting activity of the factor is nearly complete within 2 min indicating extreme rapidity of MIP action. The factor does not lose its activity even when heated at 100°C for 5 min. The data show that MIP is heat stable (data not shown). The effect of pH on the activity of MIP was shown in Figure 7. Sperm forward motility-promoting potency of MIP was nearly maximal at pH 8.0. There was no forward motility in absence of MIP. MIP has little efficacy to initiate forward motility at neutral pH (pH 7.0). MIP as well showed its efficacy to augment forward motility of mature cauda-sperm. MIP (80 μg/ml) enhanced forward motility of mature goat sperm from the initial 15 ± 0.6% to 37 ± 0.5%, that is, to the extent of nearly 140%. Treatment of caput-sperm with isolated MIP elevated significantly the intrasperm level of cyclic AMP (Table 2).

Protein specificity

It is possible that the observed motility-promoting activity of MIP may be due to non-specific action of the protein. Studies were therefore carried out to estimate the forward

TABLE 1. Purification of motility initiating protein from goat epididymal plasma

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Caudal EP	2,396	24,000	10	100	1
30–70% $(NH_4)_2SO_4$	763	62,400	82	260	8
DEAE-HPLC	169	48,600	288	220	29
ConA-Sepharose	23	15,840	680	66	68
Chromatofocusing	3	4,800	1,561	20	156
Alumina gel	1	3,120	2,437	13	243
Sephacryl S-200 gel filtration	0.6	3,000	5,000	12.5	499

Motility initiating protein was isolated from 250 ml of goat caudal epididymal plasma by the procedure described in Materials and Methods Section.

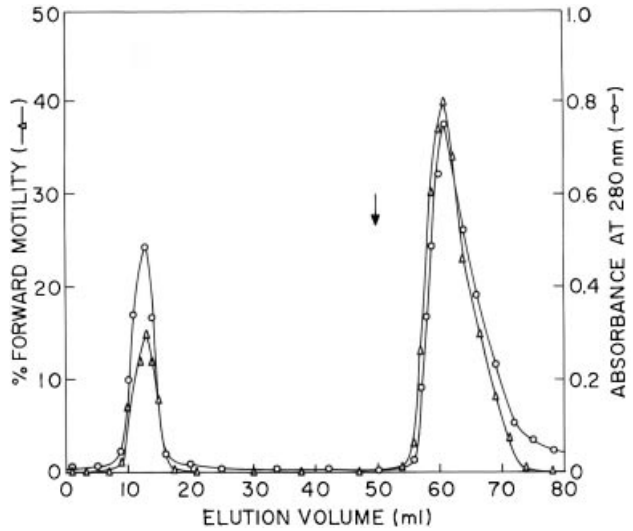

Fig. 1. Affinity chromatography of MIP on Concanavalin A-Sepharose. An extensively prewashed 10 ml column (1.0 cm × 12 cm) of Concanavalin A-Sepharose was equilibrated with buffer-I. Active MIP fraction eluted from DEAE-HPLC column and subsequently dialyzed against buffer-I was subjected to affinity column as described in Materials and Methods Section.

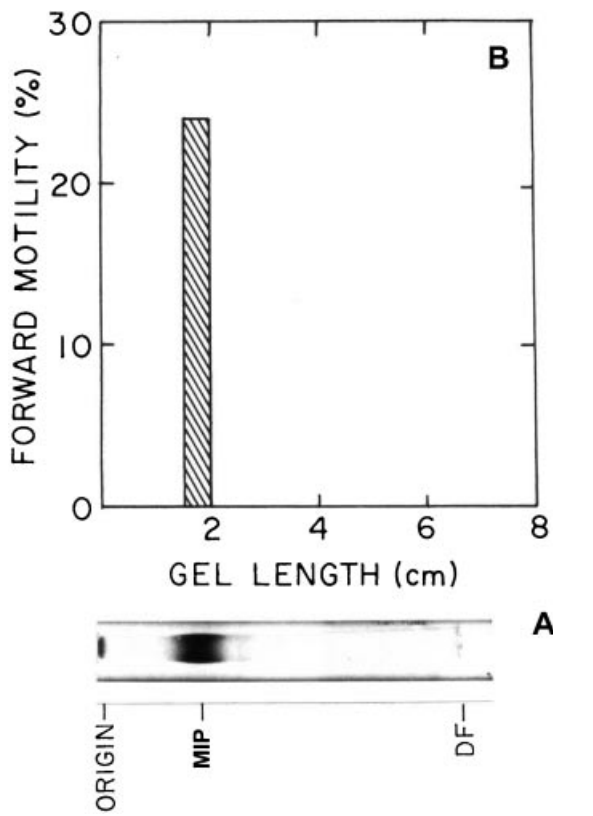

Fig. 2. Non-denaturing polyacrylamide gel electrophoresis of the purified MIP. Gel electrophoresis, activity elution from gel and staining of gel was carried out as described in Materials and Methods Section. A: Gel electrophoresis (6%) pattern of MIP. B: MIP activity measured in the gel slices.

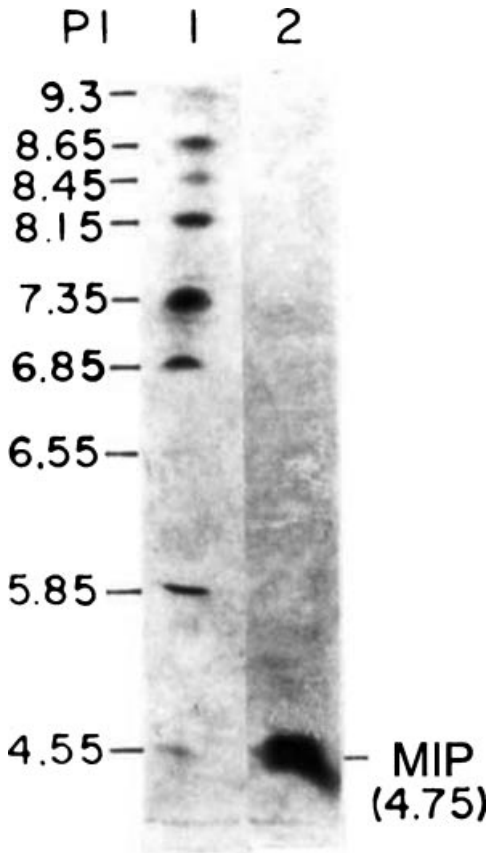

Fig. 3. Analytical isoelectric focusing of MIP. PI calibration marker proteins and purified MIP were subjected to analytical isoelectric focussing using 5% acrylamide gel containing pharmalyte, pH 3–10, as described in Materials and Methods Section. The PI calibration kit was made up of the following markers. (1) Trypsinogen (PI 9.30), (2) lentil lection basic band (PI 8.65), (3) lentil lectin middle band (PI 8.45), (4) lentil lectin acidic band (PI 8.15), (5) myoglobin basic band (PI 7.35), (6) myoglobin acidic band (PI 6.85), (7) human carbonic anhydrase B (PI 6.55), (8) bovine carbonic anhydrase-B (PI 5.85), and (9) soyabin trypsin inhibitor (PI 4.55).

motility-promoting activity of several commercially available proteins such as fetuin, ovalbumin, and BSA (Table 3). Fetuin, ovalbumin, and BSA at a concentration as high as 800 μg protein/ml induced forward motility to only 2–3% of the sperm cells. These proteins at the level of 30 μg/ml had no detectable effect on sperm motility (data not shown). However, MIP at a concentration as low as 30 μg protein/ml showed maximal activity when it induced forward motility in nearly 36% of caput-sperm indicating that MIP has high protein specificity. Immunological protein specificity of these commercially available proteins were also tested by ELISA (data not shown). Fetuin has been found to give weak immunoreaction with MIP antibody in ELISA test while BSA and ovalbumin did not show any cross-reactivity with MIP antibody. The data suggest that fetuin shares some common epitopes with MIP, which cross-reacts with MIP antibody. The results support the notion that MIP is a specific protein.

Western blotting

As shown in Figure 8A only one stained band was obtained on nitrocellulose membrane upon Western blotting of the crude

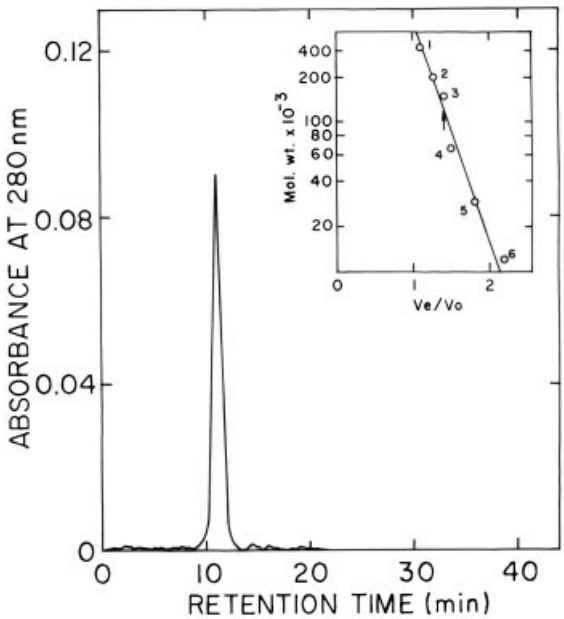

Fig. 4. HPLC gel filtration profile of purified MIP. The purified MIP was chromatographed on TSK-G3000 SW column (7.5 mm × 3 cm) using the waters HPLC system. The column was previously equilibrated with the RPS medium at a flow rate of 0.8 ml/min. Protein elution was monitored at 280 nm. The MIP activity was eluted as a sharp peak and its molecular weight was determined from the plot shown in the inset. Molecular weight calibration of the column was done by using the standard proteins. The standards used were: (1) apoferritin (443 kDa); (2) β-amylase (200 kDa); (3) alcohol dehydrogenase (150 kDa); (4) bovine serum albumin (66 kDa); (5) carbonic anhydrase (29 kDa); and (6) cytochrome c oxidase (142 kDa). The log molecular weight (in kDa) was plotted against Ve/Vo (inset), where Ve is the elution volume of each protein and Vo is the void volume of the column.

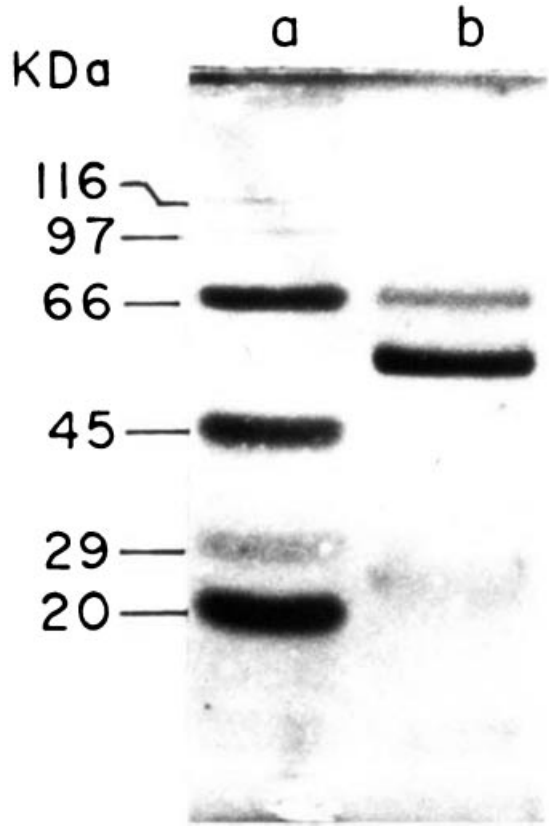

Fig. 5. SDS–PAGE using 10% polyacrylamide gel. Markers used were β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa).

epididymal plasma after non-denaturing PAGE. The stained band corresponds to the position of the native MIP thereby demonstrating that antibody has high immunological specificity only for MIP. Purified MIP was subjected to SDS–PAGE and on Western blotting showed two stained bands whose molecular weight are 70 and 54 kDa. These bands correspond to the two subunits of MIP as demonstrated earlier by SDS–PAGE in Figure 5. The result thus demonstrates that MIP antibody recognizes both the subunits (Fig. 8B).

MIP is a glycoprotein

As shown in Figure 1, MIP binds to Con A-Sepharose and it can be eluted with 0.5 (M) α-methyl-D-mannopyranoside indicating thereby that MIP is a glycoprotein having at least one D-mannose and/or D-glucose residue at its sugar side chain. Lectin specificity of purified MIP was tested by Ouchterlony double diffusion system using different lectins such as Con A, WGA, RCA2, and kidney bean lectin (Fig. 9). A distinct precipitation line appeared only with Con A. The results clearly indicate that MIP is a glycoprotein that interacts with Con A with high affinity. Sugar residues of MIP were analyzed by gas liquid chromatography (Fig. 10). MIP contains mannose, galactose, and *N*-acetylglucosamine in the ratio of 6:1:6.

Action of glycosidase on MIP

Since MIP was found to be a glycoprotein, it was treated with various glycosidases in an effort to investigate whether sugar

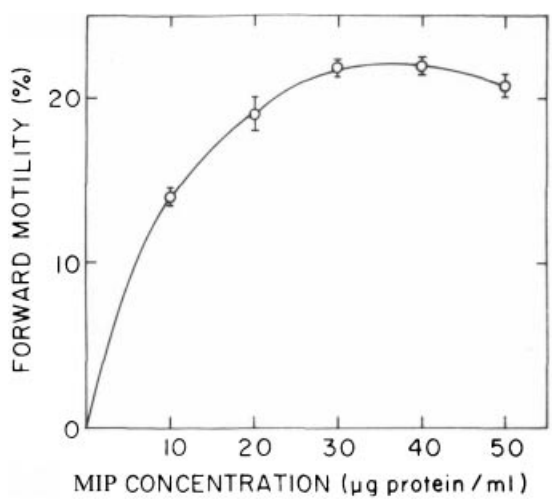

Fig. 6. Dose course of MIP for initiation of forward motility in caput-sperm under the standard assay conditions.

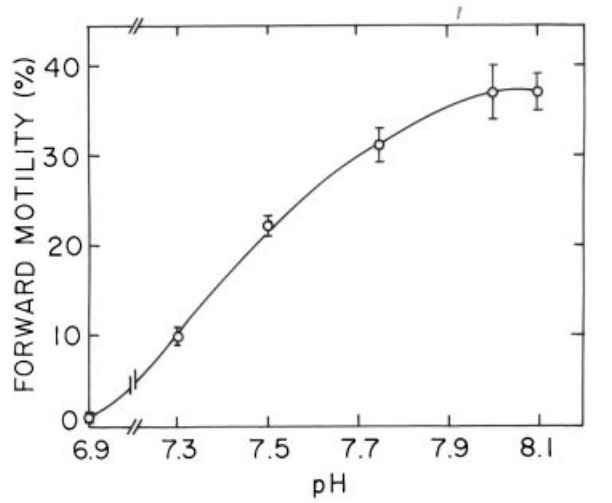

Fig. 7. Effect of pH on the activity of MIP. Standard assay conditions were used except for the variation in pH of the RPS medium. Amount of MIP used was 30 μg/ml. The data shown are mean ± SEM of three experiments.

residues are responsible for the MIP activity (Table 4). Treatment with α-mannosidase (27 U/ml) and β-*N*-acetylglucosaminidase (1.6 U/ml) inhibited completely the activity of the factor. While other glycosidases such as β-galactosidase, α-L-fucosidase, α-glucosidase, neuraminidase, and glucose oxidase did not have any appreciable effect on the MIP activity. The data show that both the sugar residues (mannose and *N*-acetylglucosamine) of MIP are essential for its activity.

Effect of MIP antibody on sperm forward motility

The effect of MIP antibody was tested on cauda-sperm forward motility as well as on caput-sperm motility induced by our in vitro initiation method described above (Fig. 11). MIP antibody (1:500 and 1:100 dilution) markedly decreased the forward motility of both caput- and caudal-sperm within 30 min of incubation. MIP antibody at a dilution of 1:100 inhibited ~90% forward motility of caput- and cauda-sperm after 30 min of incubation. The control rabbit serum from non-immunized rabbit did not have any significant effect on the sperm forward motility. There was no detectable sperm agglutination by MIP antibody under the conditions of the experiment implicating thereby that the observed motility inhibition by the antibody is not due to sperm agglutination.

Tissue distribution of MIP

The levels of MIP in tissue extracts of different organs of goat such as liver, lung, spleen, heart, kidney, bone marrow, and cauda-epididymis and have been investigated by ELISA (Fig. 12). Of all the tissues tested, bone marrow showed the highest level of MIP and it was followed by cauda-epididymis. MIP level was rather low in the other tissues. The concentration of MIP in epididymal fluid was remarkably high. The level of MIP in epididymal plasma was ~15-fold higher than that in bone marrow.

TABLE 2. Effect of purified MIP on cyclic AMP content of goat caput-sperm

Treatment	Cyclic AMP concentration (pmol/10^9 cells) mean ± SEM
Control	144 ± 9
+MIP (6 nM)	203 ± 16*

The control assay system contained caput-sperm incubated in RPS medium containing 30 mM theophylline. Cyclic AMP content of sperm incubated in presence or absence of MIP has been measured as described in Materials and Methods Section. The data shown are mean ± SEM of five experiments.
*$P < 0.05$ when compared with control.

TABLE 3. Effect of different proteins on initiation of forward motility in the immature goat caput-sperm

Additions	% forward motility mean ± SEM
Control[a]	0
+MIP (30 μg/ml)	36 ± 4
+Fetuin (800 μg/ml)	2 ± 1
+Ovalbumin (800 μg/ml)	3 ± 0.6
+BSA (800 μg/ml)	3 ± 1.1

[a]Control assay system contains caput-sperm incubated in 250 μl of RPS medium, pH 8.0, containing 30 mM theophylline and ASF (250 μg protein/ml). The data shown are mean ± SEM of three experiments.

Discussion

As reviewed in the Introduction Section, earlier investigators have reported the occurrence of multiple sperm motility regulating undefined protein factors in the male reproductive fluids (Morton and Chang, 1973; Gaur and Talwar, 1975; Acott and Hoskins, 1978; Hoskins et al., 1978; Sheth et al., 1981; Pinto et al., 1984; Jaiswal and Majumder, 1998; Ding et al., 2007; Tso and Lee, 2008). However, none of these motility proteins have been purified to apparent homogeneity and characterized. The present study reports for the first time the purification of a motility-promoting protein to apparent homogeneity from a male reproductive fluid (epididymal plasma) using the caprine model (Table 1). Some of the physical, biochemical, and functional characteristics of MIP have also been investigated. The homogeneity of MIP isolated from goat epididymal plasma, has been confirmed by multiple fractionation techniques—native PAGE, isoelectric focusing, HPLC, etc. (Figs. 2–4, S2, S3).

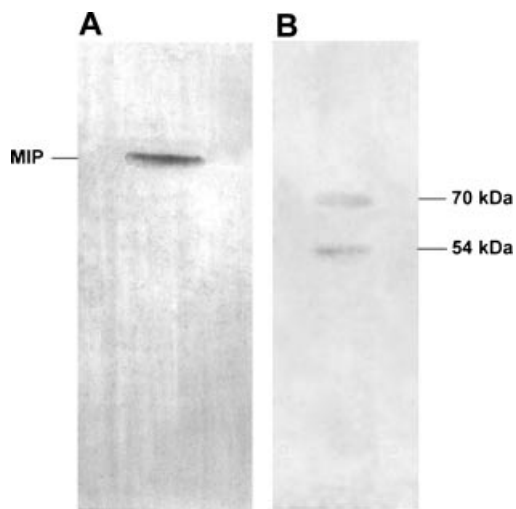

Fig. 8. Determination of antibody specificity by Western blotting as described in Materials and Methods Section. A: Western blotting of epididymal plasma (60 μg) after 6% non-denaturing PAGE. B: Western blotting of purified MIP (40μg) after 10% SDS–PAGE.

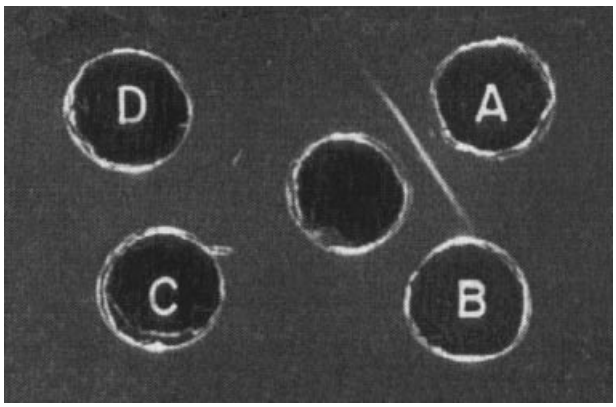

Fig. 9. Ouchterlony double diffusion test using different lectins: central well, purified MIP (20 μg). Amount of lectin used per well is 50 μg: A, Con A; B, WGA; C, RCA_2; D, kidney bean lectin.

It is an acidic protein as its isoelectric point is 4.75. It is a glycoprotein and its sugar side chain being essential for its activity (Figs. 1,9 and 10 and Table 4). It has little activity at neutral pH but it is potentially active at alkaline pH (pH 8) (Fig. 7). This finding provides biochemical basis of the earlier observation that external alkaline pH favors forward motility initiation in the immature caput-sperm in vitro in presence of crude epididymal plasma (Jaiswal and Majumder, 1998).

The native molecular weight of MIP as estimated by Sephacryl S-200 gel filtration, native PAGE and HPLC (Figs. 4 and S2) is ~124 kDa. MIP when subjected to denaturing SDS polyacrylamide electrophoresis, resolves into two protein bands: 70 and 54 kDa that were detected by the silver staining method of Wray et al. (1981) (Figs. 5 and S4). Both the protein bands are well defined and sharp, the band intensity of the 54 kDa band being much more prominent than the 70 kDa-band. If 70 kDa protein is a contaminant, MIP will be a homopolymer of 54 kDa protein. But based on simple mathematical calculation this is not possible as 124 kDa (native molecular weight of MIP) cannot be a polymer of 54 kDa. The summation of the molecular weights of the above mentioned two protein bands: 54 and 70 kDa is same as the native molecular weight of MIP. Considering the native molecular weight of 124 kDa, the only possible subunit structure of MIP is that it is a heterodimer made up of two subunit: 54 and 70 kDa at molar ratio of 1:1. As reported by several investigators (Merril et al., 1982; Gianazza et al., 1984; Merril and Pratt, 1986; Wedrychowski et al., 1986) and subsequently reviewed by Syrovy and Hodny (1991) and Walker (2002), although silver-stain methods have great sensitivity, they are extremely problematical for quantification purposes. Different proteins may vary widely in their stainability. Some do not stain at all, others may show a metachromatic effect. The apparent anomaly of the silver-staining pattern of the subunits may thus be due to some inherent limitation of the silver staining method. The finding that carbohydrate content may modulate the silver-staining pattern of a protein (Liu et al., 2005), provides further support to the above contention, as MIP is a glycoprotein. The goat MIP is strongly immunogenic and immunologically specific (Fig. 8A). The MIP antibody has specificity for both the subunits (Fig. 8B).

The molecular mass of the motility-promoting protein partially purified from bovine seminal plasma (Acott and Hoskins, 1978) was 37 kDa, which is markedly lower than that (125 kDa) of purified MIP derived from caprine epididymal plasma (Figs. 4 and S2). Biochemical basis of this marked variation of the molecular mass of MIP is not clear. It may be due to species variation or due to proteolytic breakdown of native MIP in bovine seminal plasma. A motility-promoting protein

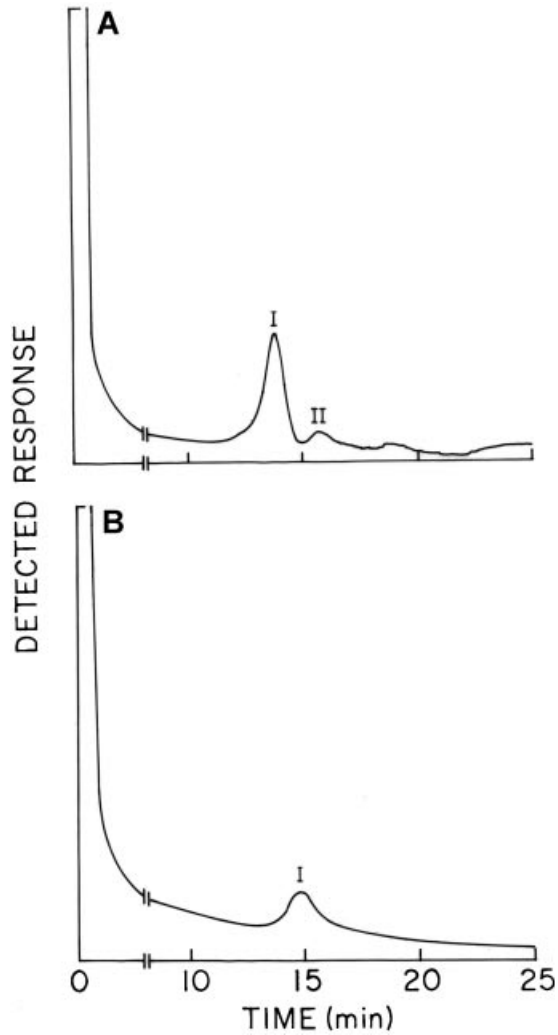

Fig. 10. Gas liquid chromatography (GLC) of sugars derived from MIP. MIP was hydrolyzed with 2 M-trifluoroacetic acid and then processed for the analysis of neutral/amino sugars. A: Detection of hexose sugars using 3% SP2340 column. I: mannose, II: galactose. B: Detection of amino sugars using 3% poly A 103 column. I: *N*-acetylglucosamine.

TABLE 4. Effect of glycosidases on MIP activity

Enzyme treatment	MIP activity (U) mean ± SEM
Nil (control)	2.0 ± 0.09
+β-galactosidase (5 U/ml)	1.9 ± 0.07
+α-glucosidase (7 U/ml)	2.0 ± 0.03
+α-mannosidase (27 U/ml)	0
+α-fucosidase (1 U/ml)	1.9 ± 0.06
+Neuraminidase (4.8 U/ml	1.8 ± 0.07
+β-*N*-acetylglucosaminidase (1.6 U/ml)	0

MIP (15 μg protein each) was pretreated with specified concentration of a glycosidase in a total volume of 100 μl of RPS medium at 37°C for 2 h. After incubation, the mixtures were heated at 80°C for 5 min to destroy the glycosidase activities and the samples were then assayed for MIP activity under standard assay conditions. The glycosidases themselves had no appreciable effect on the sperm forward motility (data not shown). The values are from three experiments.

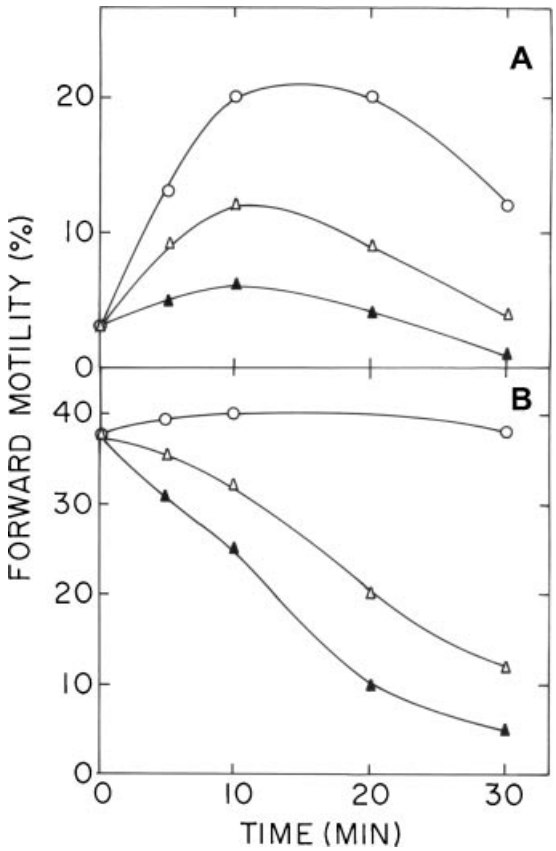

Fig. 11. Effect of MIP antibody, on inhibition of forward motility of caput (A) and cauda (B) sperm. Sperm incubated with preimmune rabbit serum 1:100 dilution (—○—) and with MIP antibody 1:500 dilution (—Δ—) and 1:100 dilution (—▲—).

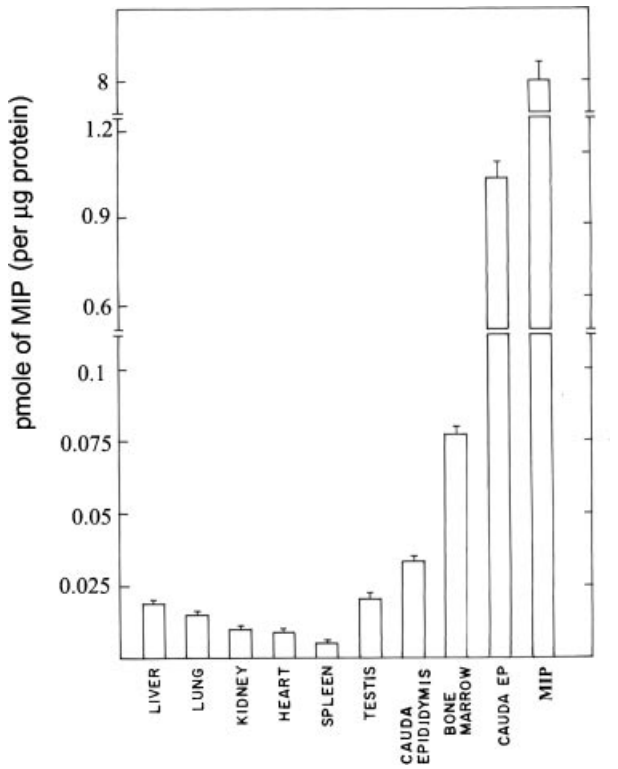

Fig. 12. Immunodetection of MIP in goat organ tissue extracts and epididymal plasma by ELISA as described in Materials and Methods Section.

(FMSF-I) has recently been purified to apparent homogeneity from buffalo serum and some of its physical and biochemical characteristics have been elucidated (Mandal et al., 2006). Like MIP, FMSF is a heat-stable glycoprotein and its sugar parts are essential for its motility-promoting potential. However unlike MIP, FMSF is a monomeric protein, much smaller in molecular size (66 kDa) and sensitive to the action of the glycosidase: α-mannosidase. In respect of tissue distribution also these two motility proteins are markedly different. Epididymal plasma is the richest source of MIP and it occurs in liver at a relatively low level (Fig. 12). Whereas reverse is the pattern in case of FMSF. The results demonstrate that MIP is clearly different from FMSF reported earlier (Mandal et al., 2006). It is also clear that although both MIP and FMSF are present in the epididymal plasma, the former is the major motility-promoting protein in epididymal plasma. The data provide further evidence to support the view that MIP is the major physiological activator of sperm motility.

Flagellar motility of the mature sperm as well as MIP-induced motility of the caput-sperm is sensitive to the action of MIP antibody (Fig. 11) strengthening thereby the view that MIP plays an important regulatory role during and following epididymal sperm maturation. These data are consistent with the view that MIP is not only a physiological initiator of forward motility in the immature epididymal sperm but also a motility activator of the mature sperm. Treatment of the caput-sperm with dialyzed epididymal plasma significantly elevated the intrasperm level of cyclic AMP in absence/presence of theophylline (Jaiswal and Majumder, 1998) and it has been speculated that MIP present in epididymal plasma induces forward motility by elevating the sperm level of cyclic AMP. This view is confirmed by the present observation that purified MIP has high efficacy to augment the intrasperm level of cyclic AMP (Table 2). Mechanism of action of MIP is still not clear. It may elevate sperm cyclic AMP level by causing decrease of the cytosolic cyclic phosphodiesterase activity and/or by activating membrane-bound/cytosolic adenylate cyclase.

Population explosion is a major problem in all developing countries. As MIP is highly immunogenic, has high immunological specificity for epididymal plasma and its antibody strongly inhibits forward motility of mature sperm, it has the potential to serve as a contraceptive vaccine. Another global social problem of immense dimension is human infertility (Hull et al., 1985). One of the reasons of human infertility is due to low order of sperm motility. As MIP has high efficacy for inducing motility in the immature spermatozoa and stimulating motility of the mature male gametes, it has great potentiality for rectifying some of the problems of human infertility utilizing various Assisted Reproductive Technologies (Boone et al., 2007). MIP as well has the potential for improving cattle breeding and preservation of endangered species. Because of the immense applied potential of this novel protein, it has recently been patented in India (Majumder and Jaiswal, 2004), USA (Majumder and Jaiswal, 2001a,b), and Japan (Majumder and Jaiswal, 2003).

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Literature Cited

Ackers GK. 1964. Molecular exclusion and restricted diffusion processes in molecular-sieve chromatography. Biochemistry 3:723–730.
Acott TS, Hoskins DD. 1978. Bovine sperm forward motility protein. Partial purification and characterization. J Biol Chem 253:6744–6750.
Albersheim P, Nevins DJ, English PD, Karr A. 1967. A method for the analysis of sugars in plant cell wall polysaccharides by gas-liquid chromatography. Carbohydr Res 5:340–345.
Boone WR, Higdon HL, Hill LK, Johnson JE, Houwing AW. 2007. Assisted reproductive technology (ART) in the upstate: Effectiveness of surgical sperm removal techniques in conjunction with ICSI to promote the success of pregnancy. J S C Med Assoc 103:8–12.
Bryan JK. 1977. Molecular weights of protein multimers from polyacrylamide gel electrophoresis. Anal Biochem 78:13–519.
Cornwall GA, Smyth TB, Vindivich D, Harter C, Robinson J, Chang TS. 1986. Induction and enhancement of progressive motility in hamster caput epididymal spermatozoa. Biol Reprod 35:1065–1074.
Davis BJ. 1964. Disc electrophoresis. ii. Method and application to human serum proteins. Ann NY Acad Sci 121:404–427.
Ding Z, Qu F, Guo W, Ying X, Wu M, Zhang Y. 2007. Identification of sperm forward motility-related proteins in human seminal plasma. Mol Repro Develop 74:1124–1131.
Ferguson KA. 1964. Starch-gel electrophoresis—Application to the classification of pituitary proteins and polypeptides. Metabolism 13:985–1002.
Gaur RD, Talwar GP. 1975. Further studies on the fertility promoting factor from human seminal plasma. Int J Fertil 20:133–136.
Gianazza E, Frigerio A, Tagliabue A, Righetti PG. 1984. Serum fractionation on immobilized pH gradients with one-and two- dimensional techniques. Electrophoresis 5:209–216.
Halder S, Dey CS, Majumder GC. 1990. Ficoll gradient isolation of immature sperm of high purity and intactness from goat epididymis. Arch Androl 24:125–128.
Hoskins DD, Brandt H, Acott TS. 1978. Initiation of sperm motility in the mammalian epididymis. Fed Proc 37:2534–2542.
Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, Coulson C, Lambert PA, Watt EM, Desai KM. 1985. Population study of causes, treatment, and outcome of infertility. Br Med J 291:1693–1697.
Jaiswal BS, Majumder GC. 1996. In-vitro initiation of forward motility in testicular spermatozoa. Int J Androl 19:97–102.
Jaiswal BS, Majumder GC. 1998. Biochemical parameters regulating forward motility initiation in vitro in goat immature epididymal spermatozoa. Reprod Fertil Dev 10:299–307.
Kann ML, Serres C. 1980. Development and initiation of sperm motility in the hamster epididymis. Reprod Nutr Dev 20:1739–1749.
Låås T, Olsson I, Söderberg L. 1980. High-voltage isoelectric focusing with pharmalyte: Field strength and temperature distribution, zone sharpening, isoelectric spectra, and pI determinations. Anal Biochem 101:449–461.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
Liu C, Erickson AK, Henning DR. 2005. Distribution and carbohydrate structures of high molecular weight glycoproteins, MUC1 and MUCX, in bovine milk. J Dairy Sci 88:4288–4294.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.
Majumder GC, Jaiswal BS. 2001a. Purified new epididymal forward motility protein and a process for the isolation of the said epididymal forward motility protein useful as a fertility promoter/blocker. US Patent No. 6,231,862; Filing Date: January 12, 1998.
Majumder GC, Jaiswal BS. 2001b. Purified new epididymal forward motility protein and a process for the isolation of the said epididymal forward motility protein useful as a fertility promoter/blocker. US Patent No 6306823; Filing Date: July 27, 1999.
Majumder GC, Jaiswal BS. 2003. Purified new epididymal forward motility protein and a process for the isolation of the said epididymal forward motility protein useful as a fertility promoter/blocker. Japan Patent No 3400367; Filing Date: Dec 24, 1998.
Majumder GC, Jaiswal BS. 2004. A process for the preparation of a purified new epididymal forward motility protein useful as a fertility promoter/blocker. Indian Patent no 189766; Filing Date: Feb 26, 1998.
Majumder GC, Mandal M. 1992. A glycoprotein factor in buffalo seminal plasma inhibits sperm forward motility. Mol Androl 3:99–114.
Majumder GC, Mandal M, Banerjee S. 2001. A novel sperm motility-promoting protein from blood serum and its applied potential. In: Ghosal SK, Ray D, editors. Recent advances in animal science research. Chandernagore, West Bengal: Orion Press International. Vol. I. pp 61–67.
Mandal M, Banerjee S, Majumder GC. 1989. Stimulation of forward motility of goat cauda epididymal spermatozoa by a serum glycoprotein factor. Biol Reprod 41:983–989.
Mandal M, Saha S, Ghosh AK, Majumder GC. 2006. Identification and characterization of a sperm motility promoting glycoprotein from buffalo blood serum. J Cell Physiol 209:353–362.
Merril CR, Pratt ME. 1986. A silver stain for the rapid quantitative detection of proteins or nucleic acids on membranes or thin layer plates. Anal Biochem 156:96–110.
Merril CR, Goldman D, VanKeuren ME. 1982. Simplified silver protein detection and image enhancement methods in polyacrylamide gels. Electrophoresis 3:17–23.
Morton BE, Chang TSK. 1973. The effect of fluid from the cauda epididymidis, serum components and caffeine upon the survival of diluted epididymal hamster spermatozoa. J Reprod Fertil 35:255–263.
Ouchterlony O. 1958. Diffusion-in-gel methods for immunological analysis. In: Kallos P, Waksman BH, editors. Progress in allergy. Basle: Karger. Vol. 5. pp 1–78.
Pinto O, Amir D, Schindler H, Hurwitz S. 1984. Effect of pH on the metabolism and fertility of turkey spermatozoa. J Reprod Fertil 70:437–442.
Rojas FJ, Bruzzone ME, Rojas IM. 1992. Regulation of cyclic adenosine monophosphate synthesis in human ejaculated spermatozoa. II. The role of calcium and bicarbonate ions on the activation of adenylyl cyclase. Hum Reprod 7:1131–1135.
Roy N, Majumder GC. 1989. Purification and characterization of an anti-sticking factor from goat epididymal plasma that inhibits sperm-glass and sperm-sperm adhesions. Biochim Biophys Acta 991:114–122.
Roy N, Majumde GC, Chakraborty CK. 1985. Occurrence of specific glycoprotein factor(s) in goat epididymal plasma that prevent adhesion of spermatozoa to glass. Andrologia 17:200–206.
Sheth AR, Gunjkar AN, Shaha GV. 1981. The presence of progressive motility sustaining factor (PMSF) in human epididymis. Andrologia 13:142–146.
Sloneker JH. 1972. In: Whistler RL, Be Miller J, editors. Methods in carbohydrate chemistry. New York: Academic Press. Vol. 6. pp 20–24.
Sluyterman L, Wijdenes J. 1978. Chromatofocusing: Isoelec-tricfocusing on ion exchanger. I. General principles. J Chomatogr 150:17–30.
Syrovy I, Hodny Z. 1991. Staining and quantification of proteins separated by PAGE. J Chromatogr 569:175–196.
Tajima Y, Okamura N, Sugita Y. 1987. The activating effects of bicarbonate on sperm motility and respiration at ejaculation. Biochem Biophys Acta 924:519–529.
Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.
Tso WW, Lee WM. 2008. Seminal plasma and progressive motility of boar spermatozoa. Int J Androl 3:243–250.
Walker JM. 2002. The protein protocols handbook. 2nd edition. USA: Humana Press. pp 237–238.
Wedrychowski A, Olinsky R, Hnilica LS. 1986. Modified method of silver staining of proteins in polyacrylamide gels. Anal Biochem 159:323–328.
Wisdom GB. 1976. Enzyme-immunoassay. Clin Chem 22:1243–1255.
Wray W, Boulikas T, Wray VP, Hancock R. 1981. Silver staining of proteins in polyacrylamide gels. Anal Biochem 118:197–203.