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Identification of Goat Sperm Ecto-Cyclic AMP Independent Protein Kinase Substrate Localized on Sperm Outer Surface

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Abstract We have demonstrated the location of a cyclic AMP independent serine/threonine protein kinase (ecto-CIK) on the outer surface of mature goat spermatozoa. We purified and characterized the major physiological protein substrate (MPS) of ecto-CIK. ^{32}P-labeled membrane proteins phosphorylated by endogenous ecto-CIK of intact cauda-epididymal spermatozoa was solubilized with 1% Triton X-100 and then fractionated by following several chromatographic techniques like Sephacryl S-300 molecular sieve chromatography, DEAE–cellulose ion-exchange chromatography and chromatofocussing. The MPS of ecto-CIK has been purified to apparent homogeneity and it was found to be a monomeric protein of 100 kDa. Three isoforms of MPS have been found with pI of 6.37, 6.05, and 5.14 and all these isoforms served as the specific substrate of ecto-CIK. The ecto-kinase has nearly 30 times greater affinity for MPS as compared to casein the most potent exogenous protein substrate. Addition of MPS (pI 5.14) antibody caused head-to-head sperm agglutination. The Fv/Fab fragment of anti-MPS caused significant inhibition of sperm motility. The data show that MPS is an ecto-protein localized on the sperm head. MPS may thus play an important role for the regulation of sperm–egg interaction. J. Cell. Biochem. 92: 164–177, 2004. © 2004 Wiley-Liss, Inc.

Key words: spermatozoa; membrane phosphoprotein; protein phosphorylation; ecto-protein kinase; sperm motility

Protein phosphorylation and dephosphorylation are important mechanisms for regulation of a number of cellular functions. The plasma membranes of mammalian cells have been shown to possess protein kinases that phosphorylate membrane associated phosphoproteins [Majumder and Turkington, 1972; Uno et al., 1977; Boman et al., 1984; Kang and Chiang, 1986; Naik et al., 1991; Lytle and Forbush, 1992; Sarrouilhi et al., 1992]. Presence of phosphoproteins have been demonstrated in the sperm plasma membrane of humans [Huacuja et al., 1997], cattle [Noland et al., 1984; Chaudhury and Casillas, 1989], hamsters [Devi et al., 1997], and goats [Halder and Majumder, 1986; Mitra and Majumder, 1991]. Bovine sperm plasma membrane have been found to possess both cyclic AMP-dependent and -independent protein kinases that cause phosphorylation of several membrane bound proteins [Noland et al., 1984]. Cyclic AMP independent protein kinase (ecto-CIK) has been shown to be localized on goat sperm plasma membrane outer surface, which causes phosphorylation of multiple outer-membrane bound phosphoproteins [Mitra and Majumder, 1991; Mitra et al., 1994]. Ecto-CIK of the isolated sperm plasma membrane phosphorylates with high affinity the serine and threonine residues of several (18, 21, 43, 52, 74, 90 kDa) endogenous phosphoproteins and the rate of phosphorylation of these proteins undergo marked modulation during epididymal sperm maturation [Nath and Majumder, 1999]. More recently the ecto-CIK has been purified to apparent homogeneity and its physiological relevance was also studied. Ecto-CIK was situated at the acrosomal tip of sperm head. It was also demonstrated that ecto-CIK plays important

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functional role in sperm physiology [PhD thesis of Nath, 1997]. But little is known about the biochemical identity of the physiological protein substrates of ecto-CIK.

The present study demonstrates, for the first time, the purification and characterization of the major physiological protein substrate (MPS) of sperm ecto-CIK.

MATERIALS AND METHODS

Reagents

The following reagents were obtained from Sigma Chemical Company (St. Louis, MO): ATP (horse muscle), sodium dodecyl sulphate (SDS) molecular weight markers, casein, dextran (average molecular weight 229 kDa), polyethylene glycol (average molecular weight 20 kDa), ethylene glycol bis-(β-amino ethyl ether) *N-N′*-tetra acetic acid (EGTA), phenyl methyl sulphonyl fluoride (PMSF), imidazole, Sephacryl S-300, β-mercaptoethanol, Triton X-100, DEAE cellulose, SDS, ammonium per sulphate, 4-chloro-1-napthol, Tween-20, nitrocellulose membrane, papain, iodoacetate, gelatin, HRP-conjugated anti-rabbit IgG, and bovine serum albumin (BSA). [γ-^{32}P]-orthophosphate (carrier free) was supplied by Bhaba Atomic Research Centre (Trombay, Mumbai). [γ-^{32}P] ATP was prepared in our laboratory according to the method described in Halder and Majumder [1986]. Poly buffer exchange-74 (PBE-74) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Isolation of Spermatozoa and Plasma Membrane

Goat epididymal spermatozoa were isolated within 2 h of slaughter [Rana and Majumder, 1987]. The cauda part of the epididymis was minced and suspended in a modified Ringer phosphate solution (RPS medium: 119 mM NaCl L^{-1}, 5 mM KCl L^{-1}, 1.2 mM $MgSO_4$ L^{-1}, 10 mM glucose L^{-1}, 16.3 mM potassium phosphate L^{-1}, 50 U penicillin ml^{-1}, pH 6.9) with gentle stirring.

Highly purified plasma membranes were isolated from the mature cauda by an aqueous two-phase polymer method [Rana and Majumder, 1989]. Membrane purity was determined by estimating marker enzymes (alkaline phosphatase, 5′-nucleotidase, acrosin, cytochrome oxidase, glucose-6-phosphatase) and by electron microscopy. The specific activities of 5-nucleotidase and alkaline phosphatase were 10–14 fold higher in isolated plasma membrane than in cell debris, indicating marked enrichment. The purity of isolated sperm plasma membrane was shown to be high as revealed by the electron microscope. The membrane preparation was finally dispersed in 25 mM potassium phosphate L^{-1} buffer, pH 7.0, containing 1 mM PMSF L^{-1}, 2 mM β-mercaptoethanol L^{-1}, 1 mM EDTA L^{-1}, 30% (v/v) glycerol and were stored at −20°C. The protein content of the plasma membrane was estimated using BSA standard [Bensadown and Weinstein, 1976].

Purification of Membrane-Bound Ecto-CIK

The ecto-CIK located on the outer surface of mature spermatozoa was purified to apparent homogeneity [Nath, 1997] following several chromatographic procedures like membrane solubilization with Triton X-100, ion exchange chromatography using DEAE–cellulose, casein–Sepharose 4B affinity chromatography, chromatofocussing, and re-affinity chromatography. Potassium phosphate buffer (5 mM, pH 7) containing 20% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1 mM PMSF L^{-1}, and 2 mM 2-mercaptoethanol L^{-1} was used throughout the purification procedure and all the steps were performed at 4°C. The purified enzyme thus obtained was used for determining the substrate specificity of the purified MPS obtained by non-radioactive procedure.

Purification of Major Physiological Substrate of Ecto-CIK

The physiological substrates localized externally were phosphorylated by endogenous ecto-CIK. The assay medium contained 5 nmol [γ-^{32}P]-ATP containing $8–10 \times 10^6$ cpm, 2 μmol $MgCl_2$ $^{-1}$L, 0.2 μmol EGTA $^{-1}$L, and 150–200 μg of plasma membrane in a total volume of 0.2 ml 50 nmole Tris-HCl L^{-1}, pH 8.5. The reaction mixture was incubated for 1 min at 37°C. The reaction was stopped with 125 μmol/L potassium phosphate buffer (pH 7), 4 μmol/L of ATP to a final volume of 2 ml water.

The labeled membrane proteins ware solubilized with 1% Triton X-100 in 5 mM potassium-phosphate buffer (pH 7) containing 1 mM PMSF L^{-1}, 1 mM EDTA L^{-1}, 2 mM β-mercaptoethanol L^{-1}, 20% (v/v) glycerol (Step 1) (Fig. 1). The solubilized labeled plasma membrane was applied to Sephacryl S-300 column (1.4×75 cm)

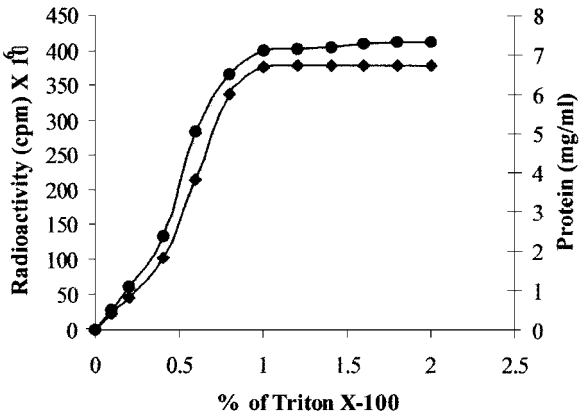

Fig. 1. Solubilization of labeled membrane with Triton X-100. Labeled membrane was solubilized with different concentration (0–2.0%) of Triton X-100. Radioactivity (◆) and protein content (●) in each case was determined.

equilibrated previously with 5 mM Tris-Cl, pH 8 containing 20% (v/v) glycerol, 1 mM PMSF L^{-1}, 2 mM β-mercaptoethanol L^{-1}, 1 mM EDTA L^{-1}, and 0.05% (v/v) Triton X-100 (Buffer A). The fractions (1 ml) were collected after void volume with monitoring ^{32}P radioactivity of each fraction (Step 2) (Fig. 2). The fractions containing the highest radioactive protein peak were then subjected to DEAE cellulose column (1×5 cm) previously equilibrated with Buffer A. The radioactive fraction was eluted by linear gradient of NaCl in Buffer A. The major radioactive peak was eluted at 60 mM salt concentration (Step 3) (Fig. 3). Fractions eluted at 60 mM NaCl in Buffer A was concentrated to a great extent and dialyzed against PBE-74–HCl (pH 4) or eluent buffer and subjected to chromatofocussing using PBE-94 (0.7×10 cm) equilibrated with 0.025 M imidazole–HCl, pH 7 [Sluyterman and Elgersma, 1978]. The elution was monitored by measuring pH as well as radioactivity of each fraction (Step 4) (Fig. 4). The three radioactive fractions were concentrated using PM-30 Diaflo ultrafiltration membrane and used for molecular weight determination, dose course and time course experiments. The third peak containing the maximum count (MPS) "substrate c" was concentrated and used in raising antibodies and related experiments. All procedures ware performed at 4°C and the concentrated radioactive fractions (including MPS) were preserved in Buffer A at −20°C (Table I).

Polyacrylamide Gel Electrophoresis in Non-Denaturing Condition

To check the homogeneity, the isolated substrates of ecto-CIK was analyzed for non-denaturing (without SDS) polyacrylamide gel electrophoresis [Weber and Osborn, 1969]. The gel was electrophoresed for 4 h with constant current of 20 mA using bromophenol blue as tracking dye (Fig. 6).

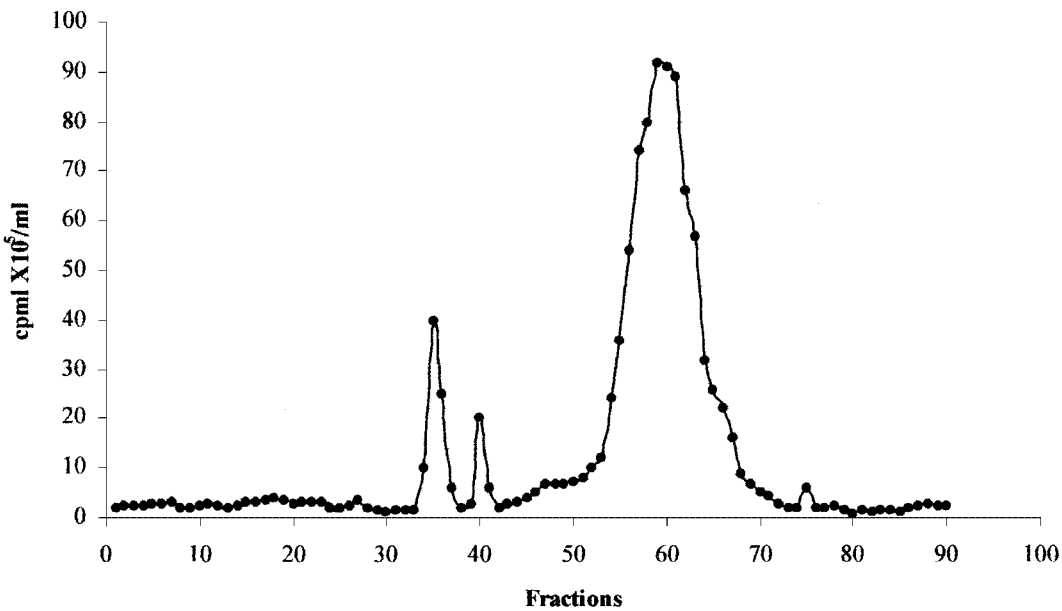

Fig. 2. Molecular sieve chromatography of solubilized ^{32}P-labeled membrane. The fraction size was 1 ml and an aliquot of each fraction was used to measure radioactivity (◆).

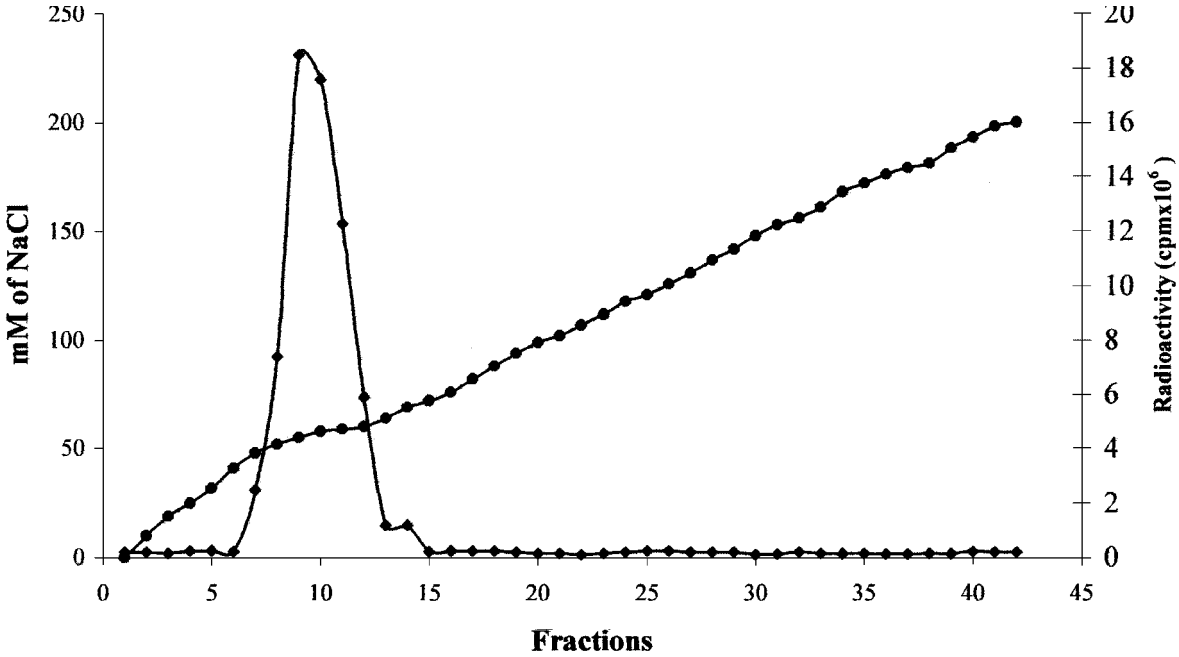

Fig. 3. Ion-exchange chromatography of the major radioactive peak obtained by gel filtration. The elution was monitored by measuring the radioactivity (◆) of each fraction and also by concentration of NaCl in Buffer A measured by the conductivity (●) of each fraction.

Determination of Molecular Weight

Molecular weight of the physiological substrates of ecto-CIK was determined by using Sephacryl S-200 gel filtration column (0.9 × 50 cm) [Wollny et al., 1984]. The gel was equilibrated with Buffer A and 50 μg of the purified substrate was loaded on the column. Elution was carried out with Buffer A at a flow rate of 3 ml/h. Fractions (1 ml) ware collected and protein was estimated by measuring the radioactivity. The column was calibrated with known molecular weight markers such as cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase b (97 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) (Fig. 5a).

Determination of Subunit Composition

To determine the subunit composition of physiological substrates of ecto-CIK, the purified substrate was subjected to SDS–PAGE Laemmli [1970]. Markers used for determination of molecular weight of subunits of substrates of ecto-CIK were β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20 kDa). Purified substrates (as obtained at chromatofocussing) each of 10 μg was loaded in three successive lanes. After completion of run the protein bands ware detected by silver staining (Figs. 5b and 6).

TABLE I. Summary of Purification of Major Physiological Substrate of Cyclic AMP Independent Protein Kinase (Ecto-CIK)

Step	Total protein (μg)	Total counts/ min (cpm)	cpm/μg of protein	Fold purification	Recovery (%)
Plasma membrane	8,032	100×10^6	0.0125×10^6	1	100
Triton extract (Step I)	6,800	90×10^6	0.013×10^6	1.06	90
Sephacryl S-300 molecular sieve chromatography–Peak c (Step 2)	52.32	73×10^6	1.39×10^6	112	81
DEAE–ion exchange chromatography (Step 3)	21.6	50×10^6	2.315×10^6	185.92	68.5
Chromatofocussing Peak 3 (Step 4)	8.4	19.792	2.36×10^6	189.236	39.58

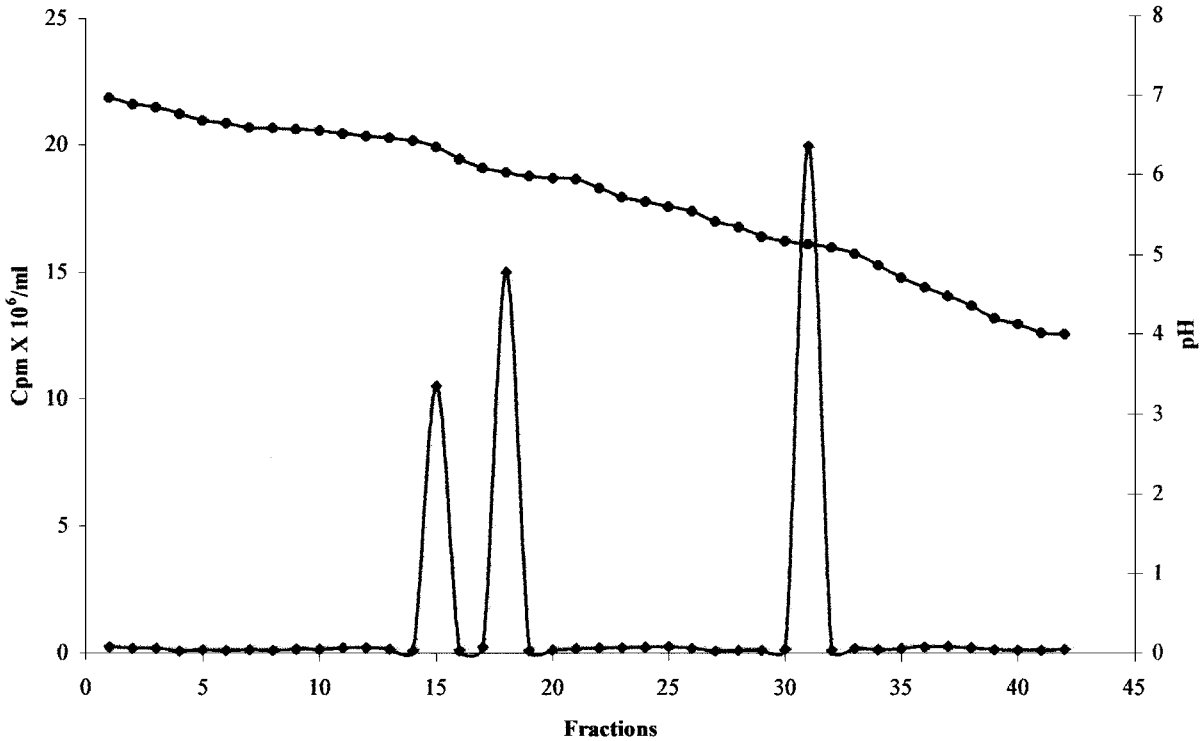

Fig. 4. Chromatofocussing of the radioactive fraction eluted from ion-exchange column using poly buffer exchange-94 (PBE-94). The elution was monitored by measuring pH (●) as well as radioactivity (◆) of each fraction.

Assay of Ecto-CIK Using Casein as the Substrate

The standard assay system contained 200 nmoles of ATP containing $20-50 \times 10^4$ cpm, 2 μmole of $MgCl_2$, 0.1 μg ecto-CIK, and 1 mg casein in a total volume of 0.2 ml of 50 mM of Tris-HCl buffer, pH 9.0. The incubation was carried out at 37°C for 5 min. The reaction was stopped by adding 0.1 ml 0.5% casein containing 250 mM potassium phosphate, 10 mM ATP, and 2 ml 10% TCA. The radio labeled protein was recovered by filtration through Whatmann No. 1 filter paper, washed with 5% TCA dissolved in scintillation fluid, and counted for radioactivity. One unit of enzyme activity has been defined as the amount of enzyme that catalyzes transfer of 10 pmole of ^{32}P from [γ-^{32}P]-ATP to substrate (casein).

Assay of Purified Ecto-CIK Using Purified MPS

The standard assay system contained 200 nmoles of ATP containing $20-50 \times 10^4$ cpm, 2 μmole of $MgCl_2$, 0.1 μg ecto-CIK, and varying amounts (0–90 μg) of purified MPS (obtained by non-radioactive procedure) in a total volume of 0.2 ml of 50 mM of Tris-HCl buffer (pH 9.0). The incubation was carried out at 37°C for 5 min. Adding 0.1 ml 0.5% casein containing 250 mM potassium phosphate, 10 mM ATP, and 2 ml 10% TCA, stopped the reaction. The radio labeled protein was recovered by filtration through Whatman No. 1 filter paper, washed with 5% TCA dissolved in scintillation fluid and counted for radioactivity. One unit of enzyme activity has been defined as the amount of enzyme that catalyzes the transfer of 10 pmole of ^{32}P from [γ-^{32}P]-ATP to substrate (casein/ physiological substrate) (Fig. 7).

Protein Estimation

Protein concentration of the sample containing glycerol and Triton were estimated [Bensadown and Weinstein, 1976] by precipitating the protein with TCA and Na-deoxycholate, prior to colorimetric assay of protein.

Autoradiography of Labeled Membrane Proteins

The purified physiological substrates from mature motile spermatozoa were dissolved in SDS–PAGE reducing sample buffer and applied to 10% SDS–PAGE. The electrophoresis

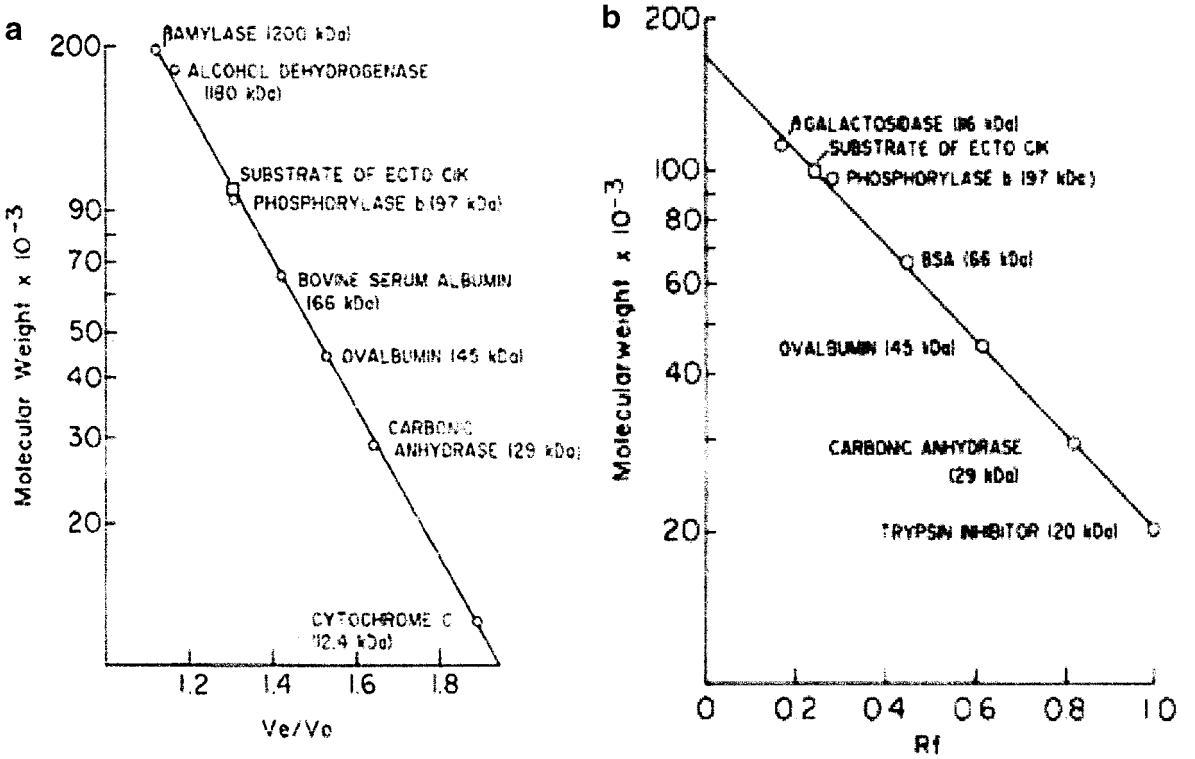

Fig. 5. Determination of molecular weight: (**a**) by Sephacryl S-300 gel filtration. Standard proteins and Blue Dextran were detected by absorption at 280 and 660 nm, respectively. The activities of the three substrates were estimated using purified ecto-cyclic AMP independent (CIK) under standard assay conditions. The molecular weight markers used as standards were β-amylase (200 kDa), alcohol dehydrogenase (180 kDa), bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). **b**: By 10% SDS–polyacrylamide gel electrophoresis. Markers were β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and trypsin inhibitor (20).

was carried out for 4 h using bromophenol blue as the tracking dye. Then the gel was dried in an electric gel drier at 80°C for 2 h. After that the gel was autoradiographed. Autoradiography was also done using the radioactive fractions obtained at the different steps of the purification procedure (Fig. 8).

Raising Antibody Against the Purified Physiological Substrate

Anti-serum against the purified MPS was raised in rabbit by four successive injections at 1st, 7th, 15th, and 21st day. First injection was given subcutaneously using 500 μg of protein in complete Freund's adjuvant. Second and third injections were comprised of 200 μg of protein in incomplete Freund's adjuvant. Fourth injection contained 400 μg of substrate in incomplete Freund's adjuvant. Blood was collected from the ear vein on 27th day of inoculation and serum was prepared and stored at −70°C. Non-immune blood serum was collected from the same animal before starting inoculation program [Ouchterlony, 1958].

The immunoglobulins of the immune serum were precipitated twice with 50% ammonium sulphate. The final precipitate was dissolved in 0.25 M PBS (pH 8.0) and dialyzed overnight against the same buffer.

Production of Monovalent Antibody

Monovalent antibody was produced by digesting the raised polyclonal antibody with papain [Harrison and Mage, 1967]. One milligram of purified anti-MPS was treated with 0.001 mg of crystalline papain in 1 ml of 10 mM sodium phosphate buffered, 0.15 M NaCl, pH 7.3 with 1 mM EDTA, and 25 mM β-mercaptoethanol. The mixture was incubated at 37°C for 1 h. Then 30 mM iodoacetamide was added and incubated at 37°C for 15 min. The mixture was then applied on CM-cellulose column equilibrated with 10 mM acetate buffer, pH 5.5 after dialyzing the sample with the same buffer. The

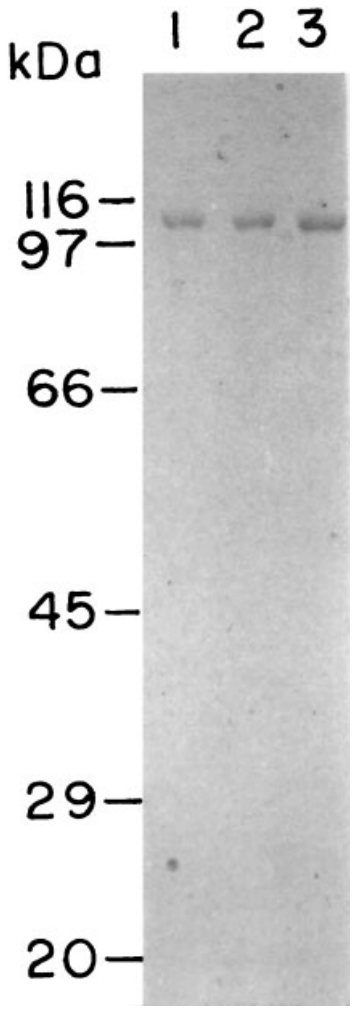

Fig. 6. SDS–PAGE of the three peaks obtained in chromatofocussing. **Lane 1**, Peak a (pI 6.37); **lane 2**, Peak b (pI 6.05); and **lane 3**, Peak c (pI 5.14). About 200 μg of protein was loaded in each lane.

Fv fragment was eluted in the unbound fraction [Porter, 1959]. The purity of the Fv fragment of anti-MPS was confirmed by performing immunoelectrophoresis [Notkins et al., 1968].

Western Blot Procedure

The antigenic specificity of the CIK antibody was evaluated by Western blot procedure. Purified substrate was run on denatured PAGE and transferred to nitrocellulose paper by "Biorad transblot cell" in a buffer containing 150 mM glycine, 20 mM Tris, 20% methanol [Towbin et al., 1979]. Non-specific binding sites were blocked with 1% gelatin in TBS (10 mM Tris-Cl pH 7.5 containing 0.9% NaCl) for 1 h at 37°C. The immobilized protein was then incubated with 1st antibody, diluted at 1:100 in TBS containing 1% gelatin, overnight at 4°C. The blots were washed in TBS containing 0.25% Tween-20, 0.1% SDS, and 0.1% gelatin and incubated in HRP-conjugated anti-rabbit IgG (2nd antibody in TBS–1% gelatin at 1:1,000 dilution) for 1 h at room temperature. After further washing, immunoreactive band was visualized using 0.05% 4-chloro-1-napthol as a substrate and 0.01% H_2O_2 in TBS, pH 7.5.

Microscopic and Spectrophotometric Assay of Sperm Motility

Percentage of forward motility was estimated by conventional microscopic method using a hemocytometer as a counting chamber. To rule out the possibility of sperm adhesion to glass, motility assays were carried out in the presence of epididymal plasma (EP) which cause nearly 100% inhibition of sperm adhesion to glass [Roy et al., 1985]. Spermatozoa (0.5×10 cells) were incubated with EP (0.6 mg protein/assay) in presence of antibody of substrate or preimmune serum at room temperature for 10, 20, 30, 60, and 120 min, respectively in a total volume of 0.5 ml of RPS. A portion of cell suspension was then injected into the hemocytometer. Forward motile (FM), motile and total number of cells were counted under a phase contrast microscope at 400× magnification.

Forward motility of spermatozoa was also estimated by a quantitative method [Majumder and Chakraborty, 1984]. The microscopic method of motility assay described above takes into consideration the number of cells with forward progression but not their velocity, whereas the spectrophotometric method was based not only on the motile cell number but also on their velocity. The method consisted of layering 50 μl of freshly extracted cauda epididymal spermatozoa (200×10^6/ml) suspended in RPS medium containing 2% Ficoll-400 at the bottom of a standard optical cuvette (3 ml capacity) containing 1.3 ml of RPS medium which was sufficient to cover the entire width of the light beam. Vigorously motile spermatozoa that moved upward and entered in the light beam were registered continuously as an increase of absorbance at 545 nm with a spectrophotometer equipped with the recorder. After reaching the maximum absorbance (A_{eq}), the content of the cuvette was mixed and the absorbance for all the cells was noted (A_t). The percentage of cells that showed vigorous forward motility was calculated as $A_{eq}/A_t \times 100$.

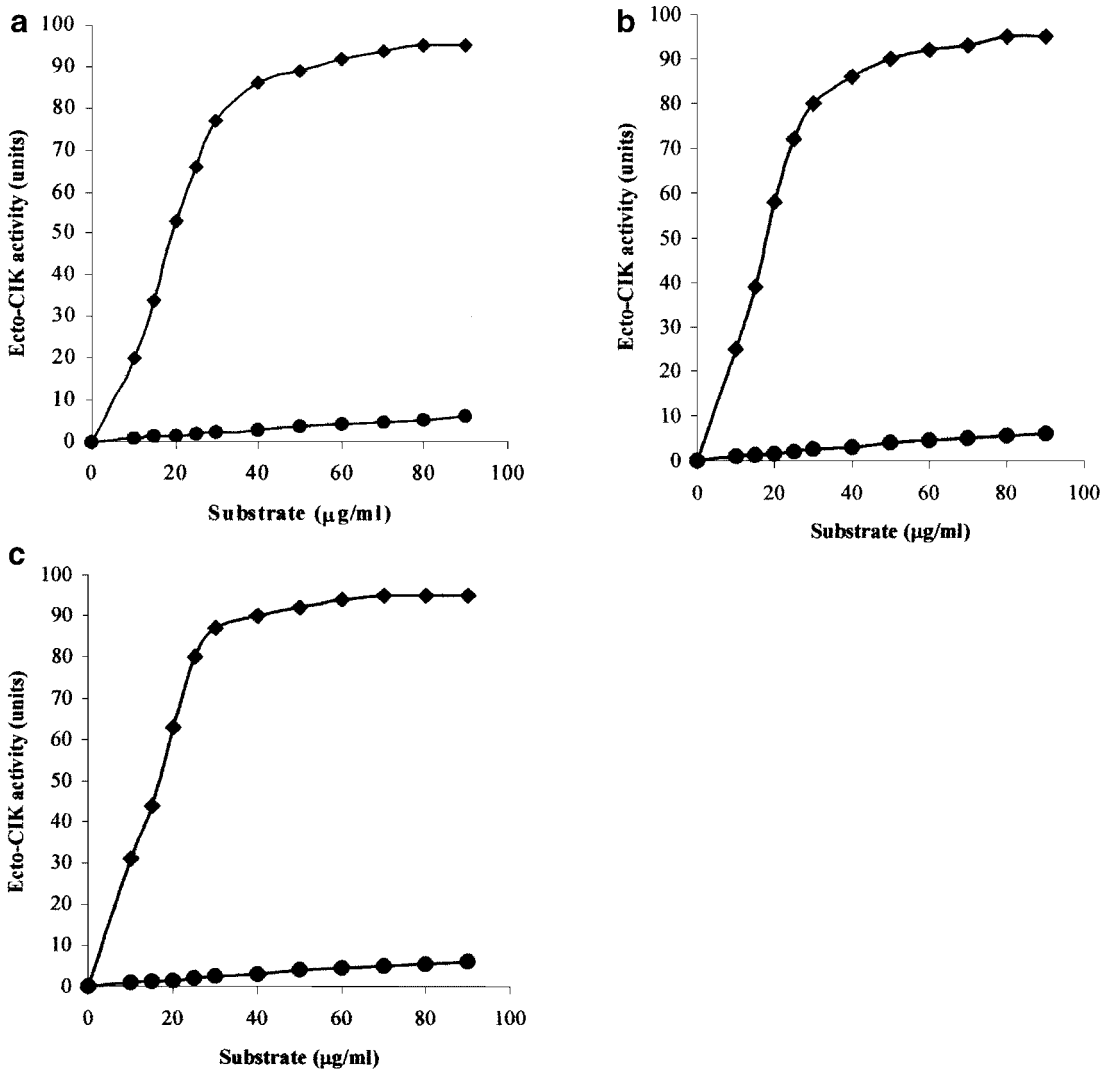

Fig. 7. Dose course of purified ecto-CIK activity against the purified substrates (the three Peaks: a–c, obtained by chromatofocussing) (◆), having c respectively was compared with the affinity of purified ecto-CIK towards casein (●), a most potent exogenous substrate.

RESULTS

Purification of MPS

Highly purified sperm plasma membrane was found to possess ecto-CIK activity that caused phosphorylation of endogenous proteins (phosphoproteins). To purify these surface substrate proteins the plasma membrane was labeled with endogenous kinases using [γ-^{32}P]-ATP. To solubilize the ^{32}P-labeled substrates, the labeled membrane was treated with different (0.1–2%) concentrations of detergent Triton X-100 (Fig. 1). The solubilization of membrane bound ^{32}P-labeled phosphoproteins increased linearly up to 1.0% and maximum solubilization was obtained at 1.0% of Triton X-100.

The Triton extract of labeled membrane then was subjected to Sephacryl S-300 molecular sieve chromatography (Fig. 2). Three ^{32}P-labeled radioactive protein peaks were obtained and among them the third (Peak 3) was the major one having 90% of total radioactivity. This major radioactive protein peak was then loaded on DEAE cellulose column and the radioactive protein peak was obtained was eluted with linear gradient of NaCl at 60 mM of salt concentration (Fig. 3). The fractions of the ^{32}P-labeled protein peaks were pooled, concentrated, and dialyzed against PBE-74–HCl buffer (pH 4) and the dialyzed fraction was subjected to chromatofocussing. Three protein peaks having pI values of 6.37, 6.05, and 5.14

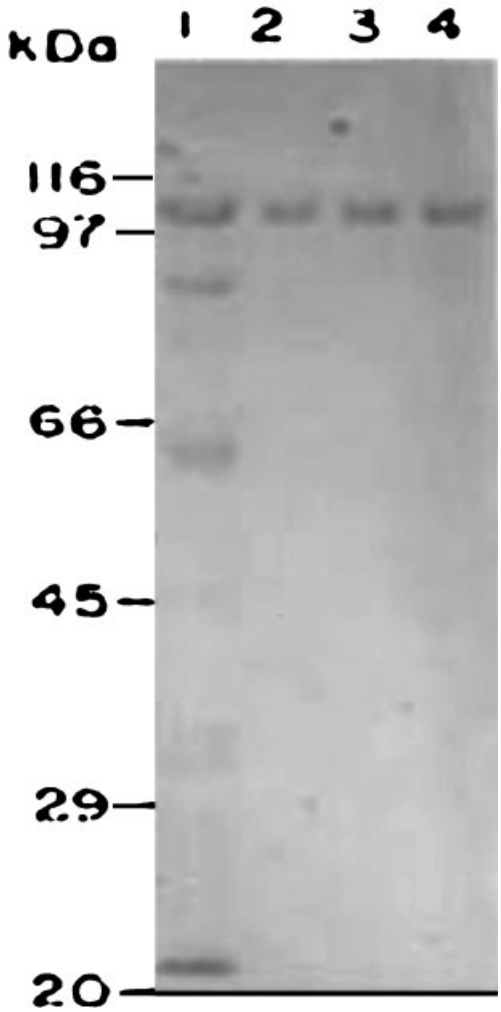

Fig. 8. Autoradiography of major physiological protein substrate (MPS) at different steps of purification. **Lane 1**, at Triton extract (Step 1); **lane 2**, at Sephacryl S-300 molecular sieve chromatography (Step 2); **lane 3**, at DEAE–cellulose ion-exchange chromatography (Step 3); **lane 4**, at chromatofocussing (pI 5.14) (Step 4). About 250 μg of protein was loaded in each lane.

were successively eluted (Fig. 4). The third protein peak having pI value 5.14 has maximum radioactivity and unless otherwise specified this protein fraction was used in subsequent studies.

Autoradiography at different steps of purification procedure demonstrated the presence of one major ^{32}P-labeled protein band of 100 kDa and a few minor bands in the soluble fraction of phosphorylated membrane. The 100-kDa band became more and more prominent with the advancement of the purification and in the last step of purification all the minor protein bands were undetectable (Fig. 8). The low molecular weight radioactive bands in the Triton extract may be the protease digestion product of the higher molecular weight substrates.

Characterization of Major Physiological Substrate

Physical properties. The isolated phosphoprotein derived from sperm outer membrane showed high degree of purity as it showed a single protein band under both native and denaturing gel electrophoresis, one peak by molecular sieving chromatography (Figs. 5b and 6) and one peak by chromatofocussing. The molecular weight of purified MPS as estimated by Sephacryl S-200 gel filtration was approximately 100 kDa (Fig. 5a). Only one peak at 100 kDa was found when the three radioactive peaks of chromatofocussing were mixed together and then applied to gel filtration chromatography column. The same molecular mass was also obtained when MPS was subjected to SDS gel electrophoresis thereby showing that it is a monomeric protein. Each of the three-chromatofocussed fractions showed molecular weight of 100 kDa when analyzed by SDS gel electrophoresis (Fig. 6). The data suggest that all these peaks represent the same protein having different levels of phosphorylation. This is due to the fact that phosphate groups carry negative charges, so variance in phosphorylation leads to the difference of in the number of negative charges and hence difference in pI was observed. When Triton extract of crude membrane was subjected to Western blot a single band was found (Fig. 10). Western blot analysis of the three purified isoforms of MPS (having different pI values) shows that three isoforms bind with anti-MPS (Fig. 9).

Biochemical properties. Purified ecto-CIK showed high affinity for purified MPS, the physiological protein substrate. The isolated enzyme has almost 30 times more affinity towards MPS as compared to casein (Fig. 7). Nearly same result was also obtained when the substrate protein of varying values were used. Ecto-CIK activity increases linearly with time up to approximately 5 min when ecto-CIK activity was measured using the purified protein substrates having different pI values (Fig. 11). The stoichiometry of phosphorylation, i.e., moles of phosphate accepting activity per mole of substrate is 1:1 (approx) in all the three cases (Fig. 7).

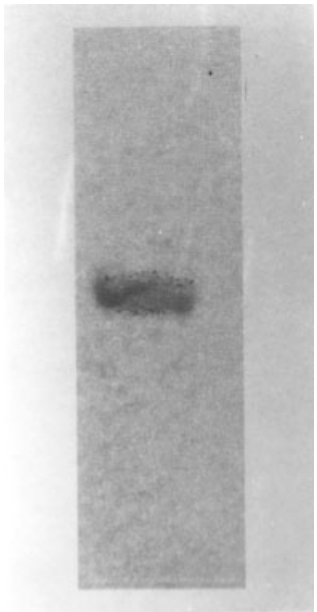

Fig. 9. Western blot of the solubilized of crude plasma membrane.

Studies With Antibodies of the MPS

Effect on cell agglutination. After incubation of the mature, motile sperm cells with anti-MPS at 37°C at antibody dilution of 1:100 head-to-head agglutination was observed under phase contrast microscope (Fig. 12).

Effect on motility. A drastic fall in motility was observed when mature motile sperm suspension in RPS medium was incubated with monovalent substrate antibody (to get rid of agglutination) at 37°C. There was inhibition of motility and forward motility in presence of MPS antibody (Fig. 13). The extent of inhibition was higher when the antibody concentration was higher. Almost same result was obtained when poly/bivalent antibody was used. The same experiment performed in presence of pre-immune sera exhibited no inhibition.

Effect of MPS antibody on specific activity of isolated CIK. Inhibition of ecto-CIK activity in presence of the substrate antibody was also observed. Ecto-CIK assay using purified ecto-CIK and purified major physiological substrate was performed in presence of different dilution of antibody. The inhibition increased with increase in antibody concentration. The same experiment performed in presence of pre-immune sera exhibited no inhibition (Fig. 14).

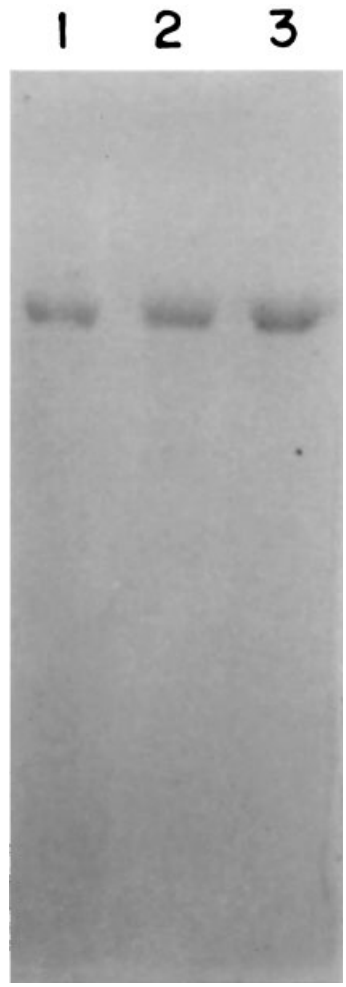

Fig. 10. Western blot of the three peaks obtained in chromatofocussing. **Lane 1**, Peak a (pI 6.37); **lane 2**, Peak b (pI 6.05); and **lane 3**, Peak c (pI 5.14).

Effect of MPS antibody on membrane phosphorylation. To prove the immunological specificity of the antibody in vitro the membrane phosphorylation experiment using endogenous ecto-CIK and [γ-^{32}P]-ATP was performed in presence of different dilution of antibody raised against the major physiological substrate. The same experiment performed in presence of pre-immune sera served as the control. The anti-MPS inhibited membrane phosphorylation and the increment if inhibition was in hand in hand with antibody concentration (Fig. 14).

DISCUSSION

Previous studies from our laboratory have provided several lines of biochemical and immunological evidences to establish the localization

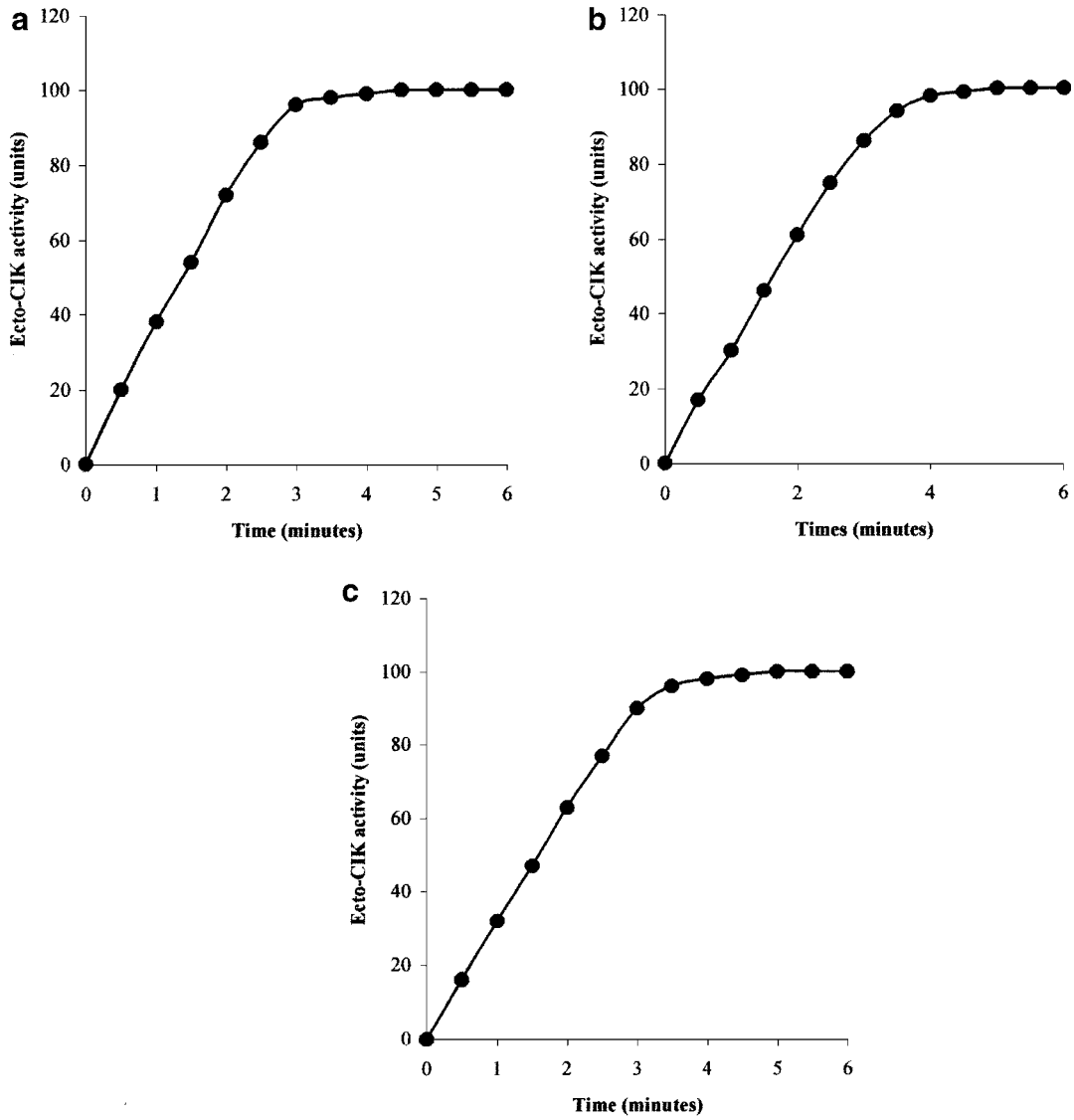

Fig. 11. Time course of purified ecto-CIK activity against the purified substrates (the three Peaks: a–c, obtained by chromatofocussing, having pI values 6.37, 6.05, and 5.14, respectively).

of an ecto-CIK on outer goat sperm surface that cause phosphorylation of several unidentified proteins [Halder and Majumder, 1986; Dey and Majumder, 1990]. An earlier study in our laboratory successfully established that ecto-CIK is functionally essential for sperm physiology as it played important role in motility regulation and acrosome reaction. Anti-ecto-CIK inhibited motility and forward motility but showed no effect on initiation of motility. Therefore, ecto-CIK was demonstrated to take part in regulation and maintenance of velocity, which is necessary for sperm forward motility. Anti-ecto-CIK also inhibited acrosome reaction and acrosin release [PhD thesis of Nath, 1997]. These are prerequisites for natural fertilization. The present study reports for the first time the purification to apparent homogeneity and characterization of the major physiological membrane-bound protein substrate (MPS) of ecto-CIK. The MPS is a 100 kDa protein and it has three isoforms differing in pI values (Fig. 4). From Western blot analysis of the three isoforms it is evident that all the isoforms binds with anti-MPS, which provide further evidence to support the view that all the three isoforms of MPS represent the same protein. MPS is the true physiological substrate of ecto-CIK as (a) it undergoes phosphorylation by intact sperm ecto-CIK and (b) it serves as a potent

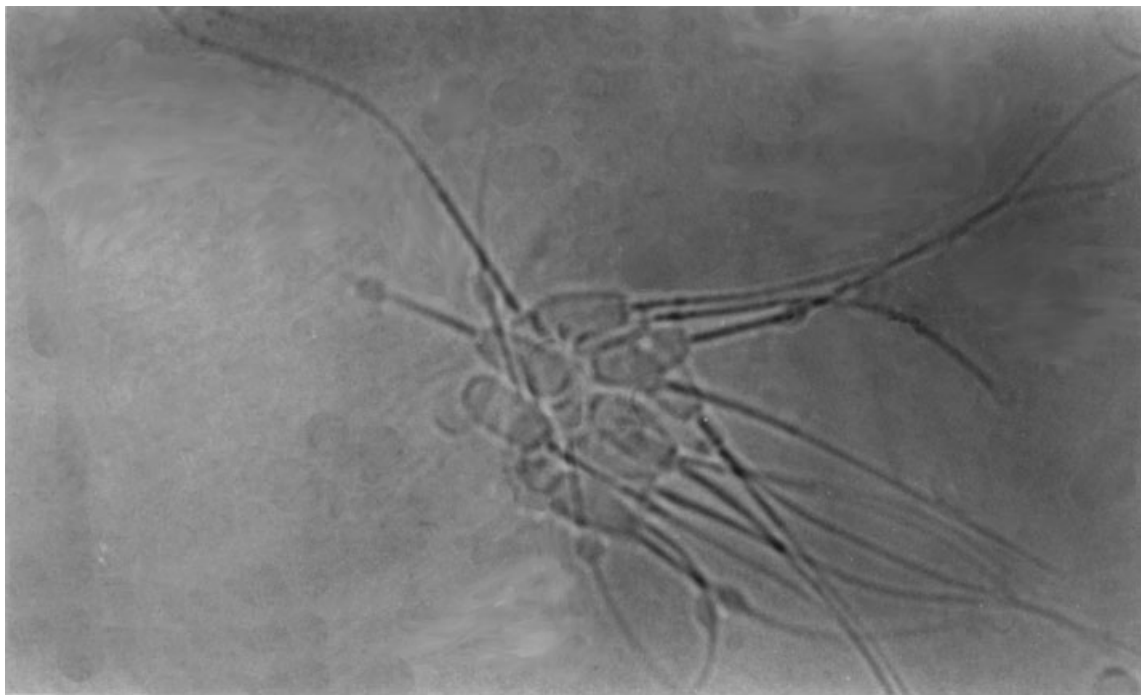

Fig. 12. Effect of MPS antibody on intact spermatozoa. Washed cells derived from cauda epididymis were with antibody at (1:100) dilution for 60 min and then visualized under phase contrast microscope at 1,000× magnification. Cells with pre-immune sera under same condition served as control (figure not shown).

substrate of the ecto-CIK whose affinity for the MPS being approximately 30 times greater than that of casein (Fig. 7). The observations that MPS serves as the protein substrate of live sperm-bound ecto-CIK and that it's antibody substantially blocks its own phosphorylation by intact sperm-bound as well as isolated protein kinase (Fig. 14) demonstrate that MPS is an ecto-protein, i.e., it is localized on sperm external surface. This finding further strengthens this view the MPS antibody mediates sperm head-to-head agglutination (Fig. 12).

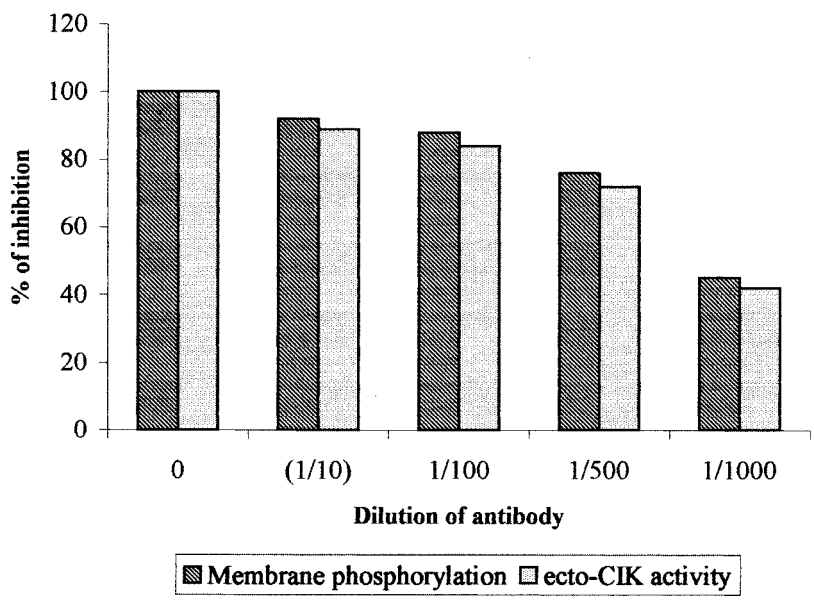

Fig. 13. Effect antibody raised against the major physiological substrate of ecto-CIK on motility and forward motility. The assays were performed under standard assay conditions.

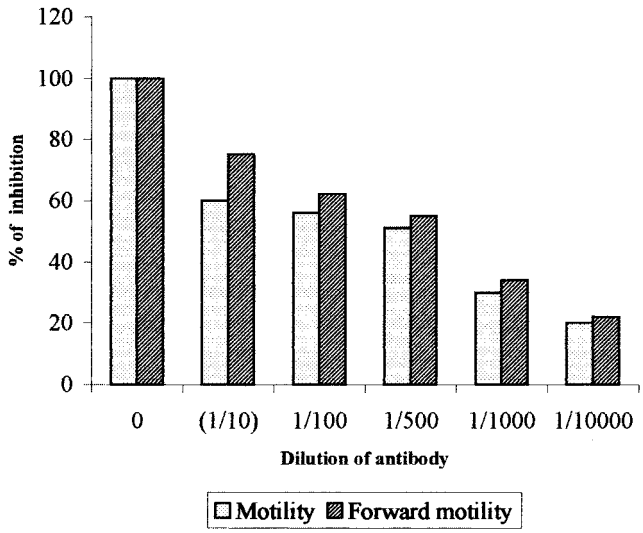

Fig. 14. Effect antibody raised against the major physiological substrate of ecto-CIK on membrane phosphorylation and ecto-CIK activity. The assays were performed under standard assay conditions.

An earlier study from our laboratory has shown that 18, 21, 43, and 52 kDa proteins are the major phosphoproteins of goat cauda sperm plasma membrane [Nath and Majumder, 1999]. In contrast to the present investigation, protease inhibitors such as PMSF, EDTA, and 2-mercaptoethanol, were not used in the buffer used for the earlier membrane phosphorylation studies [Nath and Majumder, 1999]. Thus the earlier failure, to detect 100 kDa phosphoprotein in the plasma membrane [Nath and Majumder, 1999] may perhaps be attributed to proteolytic breakdown of this protein.

Sea urchin sperm plasma membrane has been shown to contain a 160 kDa phosphoprotein which undergoes dephosphorylation when it comes in contact with egg jelly. So the membrane bound phosphoproteins may have an important role in fertilization [Ward and Vacquier, 1983]. Goat spermatozoa have been shown to possess a coupled-enzyme system, consisting of a CIK and a phosphoprotein–phosphatase, that determines the phosphorylation status of cell surface phosphoproteins by protein phosphorylation–dephosphorylation [Barua et al., 1990]. The observation that antibody of MPS inhibits its efficacy to serve as substrate of ecto-CIK as well as sperm motility (Figs. 13 and 14), shows that MPS has an important role in the regulation of sperm flagellar motility. It is of interest to note that MPS antibody causes sperm head-to-head agglutination (Fig. 12). The data are consistent with the view that MPS is localized on the sperm acrosomal cap. It is well documented that sperm acrosomal region of sperm head directly participates in binding to the egg surface. It thus appears possible that phosphorylation and dephosphorylation of MPS may constitute a major mechanism for the regulation of sperm acrosomal reaction and sperm–egg interaction.

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