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Characterization of arylsulphatase A in a 70 kDa protein isolated from goat spermatozoa having Na^+, K^+-ATPase inhibitory activity

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A protein having inhibitory effect on Na^+, K^+-ATPase as well as showing arylsulphatase A activity (ASA) was isolated from the cytosolic fraction of goat spermatozoa and characterized biochemically. The molecular mass of the protein was found to be 70 kDa (P70) on 10% SDS-PAGE after 35% ammonium sulphate precipitation, followed by hydroxyapatite column chromatographic separation. The isoelectric point (pI) of the protein was found to be 4.9. The sequencing results of first ten N-terminal amino acid residues of protein showed 100%, 90%, and 80% homology with N-terminal 18-27 amino acid residues of mice, pig and human testicular ASA, respectively. The optimum *p*H, temperature and incubation time for maximum ASA activity of the protein was 5.5, 37°C and 30 min respectively. The ASA activity of protein and AS from a commercial source was studied with respect to the sensitivity to different metal ions, vanadate, carbonyl compounds and ascorbate. Inhibition of AS activity of P70 by silver nitrate suggested that it was related to ASA. Comparable effects of different polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic acids) and purified anti P70-antibody on P70 and AS from commercial source were observed. The findings suggested that protein was novel in nature, having both regulatory and catalytic functions and showed similarities with the ASA reported from different sources.

Keywords: Arylsulphatase A, Goat spermatozoa, Inhibitor of Na^+, K^+-ATPase, 70 kDa Protein

Arylsulphatase (AS) which hydrolyze aromatic sulphates to the corresponding alcohol and sulphate has been identified as a mixture of two to three distinct forms of the enzymes (arylsulphatase A, B and C)[1-5] in several mammalian tissues. Arylsulphatases A and B (ASA an ASB) are found in the lysosomes and C in the microsomal fraction[6]. ASA is known as a lysosomal/acrosomal enzyme with a molecular mass of 65-68 kDa. The mannose residues on the saccharide moieties of ASA are phosphorylated and are responsible for targeting to the lysosomes via the binding to mannose-6-phosphate. ASA desulphates small artificial substrates e.g. *p*-nitro catechol sulphate (NCS) as well as detergent or saposine B solubilized natural sulphoglycolipids i.e. sulpho-galactosyl ceramide (SGC) and sulphogalactosyl-glycerolipid (SGG)[7-9]. ASA maintains the balance of SGC in the neurological system and the individuals genetically-deficient in ASA show SGC accumulation in the nervous tissues, thus causing dementia and paralysis, a syndrome known as metachromatic leukodystrophy[10].

ASA binds to SGG with high affinity in the absence of a detergent or saposine B, although the binding does not result in SGG desulphation[11]. The high affinity between these two molecules may also explain their co-localization in the sperm head[12,13]. Since SGG is also engaged in zona pellucida (ZP) binding, ASA and SGG may act together as complexes in this binding process. Evidences reveal that SGG is synthesized in the spermatozenic cells and then target to their plasma membranes. The level of SGG remains stable during spermiogenesis, sperm maturation and initial phase of sperm capacitation[14-16]. The substrates of the various ASs are all located in or at the exterior surface of the plasma membrane of

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Abbreviations: AS, arylsulphatase; BSA, bovine serum albumin; BCIP, bromochloroindolyl phosphate; DHA, docosahexenoic acid; EPA, eicosapentenoic acid; DTE, dithioerythretol; EGTA, ethylene glycol-bis (2-amino-ethylene)-N,N,N',N'-tetraacetic acid; βME, β-mercaptoethanol; NBT, nitroblue tetrazoline; NCS, 4-nitrocatecholsulphate; PMSF, phenylmethylsulphonyl fluoride; PUFA, polyunsaturated fatty acid; TLCK, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride; TPCK, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone; SGC, sulpho-galactosyl ceramide; SGG, sulphogalactosylglycerolipid; TBS, Tris-buffer saline;. ZP, zona pellucida.

spermatozoa and desulphation diminish the negative surface charge of the plasma membrane - a hallmark of capacitation[17]. Thus, the presence of ASs in the spermatozoa may play a key role in the process of capacitation and introduction of acrosome reaction.

Na^+, K^+-ATPase is an important enzyme in the animal cell plasma membrane[18]. The partially purified AS from pig kidney inhibits oubain sensitive Na^+, K^+-ATPase completely[19]. Polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibit AS activity[20]. Fish oil containing EPA and DHA prevents altered α2 isoenzyme activity of Na^+, K^+-ATPase in diabetic cardiomyopathy[20]. The PUFAs also affect the activity of Na^+, K^+-ATPase *in vitro*.

Although the occurrence of ASs in mammalian testes is well known, the AS activity associated with a protein (P70) in goat spermatozoa showing Na^+, K^+-ATPase inhibitory activity has not been reported. Recently, a low molecular protein from goat spermatozoa having stimulatory effect on Mg^{2+}-independent Ca^{2+}-ATPase activity has been reported from our laboratory[21]. In the present study, we report the biochemical characterization of ASA in a 70 kD protein, isolated from goat spermatozoa and having Na^+,K^+-ATPase inhibitory activity.

Materials and Methods

Materials

Hydroxyapatite, EDTA, ethylene glycol-bis(2-amino-ethylene)-N,N,N',N'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride (TLCK), 4-nitrocatecholsulphate (NCS), arylsulphatase (type VIII) from abalone entrails, eicosapentenoic acid (EPA), docosahexenoic acid (DHA), bovine serum albumin (BSA), dithioerythretol, iodoacetamide, Ponceau-S, sodium pyrophosphate, molecular weight markers, orthovanadate, alkaline phosphatase conjugated goat anti-rabbit IgG were obtained from Sigma Chemical Co., St. Louis, USA. Other chemicals of analytical grade were purchased from Sisco Research Laboratory, Spectrochem Pvt. Ltd. and Merck, India.

Purification of protein

Goat testes collected from the local slaughter house immediately after sacrificing the animals were brought to the laboratory on ice. All the subsequent procedures were carried out at 4°C. The cauda region was separated and minced with buffer A (50 mM Tris-HCl, *p*H 7.5 containing 0.25 M sucrose, 1 mM each EDTA, EGTA and PMSF, 2 mM βME , 0.1 M TPCK, 0.1 M TLCK). The sample was homogenized for 5 min in a motor driven mechanical homogenizer and centrifuged at 12,000 × *g* for 15 min. The supernatant was collected and centifuged at 100,000 × *g* for 1 h in Hitachi ultracentrifuge (Model 55P-72). Post-100,000 × *g* supernatant (cytosol) was used as source of the protein[22].

The clear supernatant was subjected to 35% ammonium sulphate precipitation, centrifuged at 25,000 × *g* for 30 min, and the pellet was dissolved in buffer-A. The suspension was dialyzed against buffer-A for 48 h with four changes and applied on to a hydroxyapatite column (4 ml) pre-incubated with 10 mM potassium-phosphate buffer (*p*H 6.8) containing 1 mM βME (buffer-B). The column was washed with buffer-B and a protein having molecular mass of 70 kD (named as P70) was eluted in buffer wash fraction. The purified protein was dialyzed against 10 mM Tris-HCl buffer (*p*H 7.5) containing 0.5 mM βME (buffer-C) for 48 h with four changes and used for subsequent experiments.

Protein estimation and SDS-PAGE

Protein was estimated according to the method of Bradford[23] using BSA as standard. SDS-PAGE and silver staining were performed according to Laemmali[24] and Morrissey[25], respectively.

Preparation of Na^+, K^+-ATPase enriched membranes and its activity assay

Na^+,K^+-ATPase enriched membranes were prepared from rat brain microsomal membranes[26] and Na^+, K^+-ATPase activity was measured[27,28]. Briefly, the reaction mixture in a volume of 0.4 ml containing 30 mM histidine buffer, 25 mM sucrose and 1 mM EDTA, *p*H 7.5, 3 mM $MgCl_2$, 20 µg protein with or without 130 mM NaCl and 20 mM KCl was incubated at 37ºC for 5 min. Reaction was initiated by the addition of 1 mM ATP and incubated at 37ºC for 30 min. It was terminated by addition of 6% ice cold trichloroacetic acid and the liberated Pi was estimated colorimetrically[28]. Na^+, K^+-ATPase activity was measured as the difference in activity between Mg^{2+}, Na^+, K^+ and Mg^{2+} alone and was about 90% sensitive to ouabain. The enzyme activity was measured in presence of P70 purified from goat testis cytosol to examine its inhibitory activity and expressed as µmoles Pi/h/mg of protein.

Arylsulphatase A (ASA) activity assay

Presence of ASA in P70 was assayed by nitrocatecholsulphate desulphation[9]. The reaction was performed at 37°C for 30 min in 200 µl of 0.5 M sodium acetate buffer (*p*H 5.5), containing 10 mM nitrocatecholsulphate, 0.5 mM $Na_4P_20_7$, 1.7 M NaCl and 2 mg/ml BSA and terminated by the addition 200 µl of 1 M NaOH. The reaction product *p*-nitrocatechol was quantified by its absorbance at 515 nm[27]. The ASA activity at different stages of purification of P70 and the activity of ASA from commercial sources was measured. The enzyme activity was expressed as µmoles product formed/h/mg of protein.

Two-dimensional gel electrophoresis

Isoelectric focusing (first dimension)

A non-linear immobilized *p*H gradient (3.0-10.0) was used as first dimension.

IPG gel strips rehydration and sample application

Hydration was performed overnight in the Pharmacia reswelling cassette with 25 ml of rehydration solution. The strips were transferred to the Pharmacia strip tray when the rehydration cassette had been thoroughly emptied. About 5 µg of P70 was suspended in IPG buffer and loaded on a reswelled immobilon *p*H gradient (IPG) strip (*p*H 3-10) (GE Bioscience) for isoelectric focusing using a multiphor apparatus. Sample was applied at the cathode end of the IPG strip in a slow and continuous manner without touching the gel. After an initial 5 h run at 500 V, isoelectric focusing was done by running the gel for 10 h at 3500 V.

SDS-PAGE

The strips were equilibrated in buffer [Tris-HCl (50 mM, *p*H 8.4), urea (6 M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v)] for 12 min followed by blocking of -SH groups with 100 ml of a solution containing Tris-HCl [(50 mM, *p*H 6.8), urea (6 M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of bromophenol blue for 5 min] and placed horizontally on a 12% SDS-PAGE. The 2D gel was then stained with silver nitrate for detection of the band[29].

N-terminal amino acid sequencing of P70

Purified P70 was loaded on to a 10% SDS polyacrylamide gel and the gel was run under normal conditions. After the run was complete, the gel was removed from the electrophoresis cell and soaked in electroblotting buffer. PVDF membrane (Immobilon PSQ, Millipore) was soaked in methanol for a few sec, following soaking in deionized water, transferred to the electroblotting solution and equilibrated for a while. Thereafter, the membrane was washed with deionized water before staining. The PVDF membrane was stained with Ponceau-S solution, washed extensively for 1 min with deionized water, air-dried and the band of P70 was excised for sequencing. The membrane was stored in a sealed 1.5 ml tube at –20°C.

N-terminal sequencing of P70 was done using an Applied Biosystems 475S sequencer at the Vanderbilt Proteomics Core Lab, USA, following Edman degradation principle for N-terminal sequencing of proteins. Ten cycles were used for ten amino acids analysis.

Effect of *p*H, incubation time and temperature on ASA activity

The effect of incubation time, temperature and *p*H on ASA activity in P70 was examined following the method described above for ASA assay, varying one of the mentioned parameters and keeping the other two fixed.

Effect of metal ions, vanadate, carbonyl compounds and ascorbate on ASA activity

Different concentrations of silver nitrate, $CuCl_2$, $FeCl_3$, vanadate, EDTA, ascorbate, hydroxyl amine and hydrazine were added to the NCS containing reaction mixture[11] and the standard assay method for ASA activity was followed[9].

Effect of EPA and DHA and their mixture on ASA and Na^+, K^+-ATPase activities

ASA activity of P70 and commercial source was measured in presence of different polyunsaturated fatty acids (EPA and DHA)[9]. Similarly, Na^+, K^+-ATPase activity was measured as described[27,28]. Two different concentrations (4 and 8 µg) of each fatty acid as well as their mixture were used.

Effect of purified anti-P70 antibody on ASA and Na^+, K^+ -ATPase activities

ASA activity of P70 as well as commercial source was measured in presence of different concentrations of anti-P70 antibody[9]. The inhibitory activity of P70 on Na^+, K^+-ATPase activity was also measured in presence of different concentrations of anti-P70 antibody following standard assay methods[27,28].

Western blot analysis

Purified P70 and commercial AS (5 µg each) were loaded on to a 10% SDS-PAGE gel for electrophoresis. After the run was complete, gel was removed and protein in gel was transferred to 0.45 µM nitrocellulose membrane (Millipore, USA). The membrane was incubated in blocking buffer (TBS containing BSA) and the blot was incubated with affinity purified anti-P70 antibody (primary antibody). After washing, the blot was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (secondary antibody). For immunodetection, bromochloroindolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) substrates were used.

Statistical analysis

Data were analyzed using mean ± S.D. from three or more experiments. Curve fitting by Microcal origin version 6.0 was used.

Results and Discussion

P70 was purified from goat spermatozoa and characterized as an inhibitor of Na^+, K^+-ATPase, isolated from rat brain microsomal membranes. Purified P70 migrated as a single band on 10% SDS-PAGE gel, as revealed from the silver staining. The purity of the protein was further confirmed from 2D gel electrophoresis and pI was calculated to be 4.9 (Fig. 1), which was comparable with the pI of ASA reported earlier[3]. Table 1 shows purification of P70 associated ASA activity from goat testis cytosolic fraction. About 50- fold enrichment of the activity was obtained in the final purified fraction.

The sequencing results of first ten N-terminal amino acid residues of P70 (Table 2) showed 100%, 90%, and 80% homology with N-terminal 18-27 amino acid residues of mice[12], pig[30] and human testicular[31] ASA, respectively. The optimum *p*H, temperature and time of incubation for maximum AS activity were 5.5, 37°C and 30 min, respectively (results not shown).

The effect by different metal ions, vanadate, carbonyl compounds and ascorbate on the ASA activity associated with P70 and in commercial source is shown in Table 3. The ASA activity of P70 and commercial source was inhibited in presence of different agents and the results were comparable with the earlier reports[32,33]. ASA activity of P70 was significantly inhibited at higher concentration of silver nitrate (0.5 mM), but no appreciable effect was observed at lower concentration, however, the effect was higher than on commercial AS. Inhibition of ASA of P70 by silver nitrate strongly suggested that it was ASA[3].

The ASA activity of P70 and commercial source decreased to about 14 and 25% respectively in presence of 0.025 mM $CuCl_2$, and 67 and 78% respectively with 0.025 mM $FeCl_3$ solution. In presence of EDTA, ASA activity from both sources remained more or less unaltered, when compared with

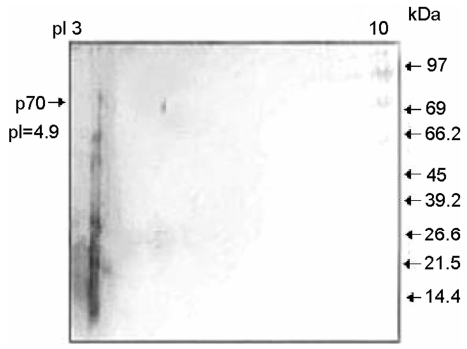

Fig. 1—Two dimensional (2-D) polyacrylamide gel electrophoresis pattern of the purified protein [Non-linear immobilized *p*H gradient (IPG strips with pI 3-10, 7 cm) was used in first dimension, the direction of the IPG strip in the diagram was from left→right. A 10% SDS polyacrylamide gel was used in the second dimension. The position of the spot corresponding to the purified protein is indicated by the arrow, the pI is calculated to be 4.9]

Table 1—Purification of P70 associated arylsulphatase A activity from goat testis cytosolic fraction

[Goat sperm was homogenized in isolation buffer (buffer A), followed by centrifugation at 12000 *g* and the supernatant ultracentrifuged at 100000 *g*. Then, the clear supernatant was subjected to 35% ammonium sulphate precipitation, followed by hydroxyapatite column chromatogram. (n = 10)]

Steps	Total protein (mg)	Total activity (µmol h^{-1})	Specific activity (µmol h^{-1} mg^{-1})	Purification (fold)
Goat testes	12.9	44.2	3.42	1
35% Ammonium sulphate	0.83 ± 0.034	37.2 ± 3.65	44.81 ± 2.26	13
Hydroxyapatite	0.19 ± 0.01	31.50 ± 2.38	170.27 ± 5.02	49

Table 2—N-terminal amino acid sequence of goat sperm P70 shown along with pig, human and mouse testicular ASA sequences reported earlier[12,31,32]

Pig	SPPNIVLIFA (18-27)
Human	RPPNIVLIFA (18-27)
Mouse	SPPNILLIFA (18-27)
Goat	SPPNILLIFA (1-10)

Table 3—Effect of metal ions, vanadate, carbonyl compounds and ascorbate on ASA activity of P70 and arylsulphatase from commercial source

[Different concentrations of $AgNO_3$, $CuCl_2$, $FeCl_3$, vanadate, EDTA, ascorbate, hydroxyl amine and hydrazine were added to the NCS containing reaction mixture and standard method for activity assay was followed. Results shown are the mean ± S.D. of four different experiments. The unit of enzyme was calculated as µmoles of 4-nitrocatechol formed per h per mg of the protein and taken as 100 percent activity i.e. "0" inhibition (control)]

Condition	Conc (mM)	Percent Inhibition	
		P70	Commercial source
None (Control)	-	0	0
$AgNO_3$	0.1	7.6 ± 0.8	3.9 ± 0.6
	0.2	20.4 ± 1.6	11.44 ± 1.4
	0.5	79.2 ± 3.8	36.8 ± 2.3
$CuCl_2$	0.025	86.2 ± 3.6	75.4 ± 2.4
$FeCl_3$	0.025	33.1 ± 1.2	22.3 ± 3.6
EDTA	1.25	0	0
	12.5	7.8 ± 1.1	0
Ascorbate	0.01	5.9 ± 0.7	3.9 ± 1.0
	0.025	13.2 ± 1.1	10.9 ± 1.4
	0.05	38.4 ± 1.9	36.7 ± 2.4
Hydroxyl-amine	1.25	51.7 ± 2.2	44.3 ± 2.3
	2.50	72.8 ± 2.8	65.4 ± 3.8
Hydrazine	12.5	56.9 ± 2.8	46.3 ± 2.8
	25.0	69.1 ± 2.6	62.3 ± 3.4
Vanadate	0.01	57.3±3.2	45.5±2.1
	0.05	74.1±2.7	66.4±2.6
	0.25	91.8±3.9	85.1±2.8

the control. In case of ascorbate, the ASA activity in both sources was affected moderately only at higher concentration, whereas vanadate, hydroxyl amine and hydrazine were found to be strong inhibitors of the ASA activity. The above findings suggested similarities between the ASA isolated from different sources.

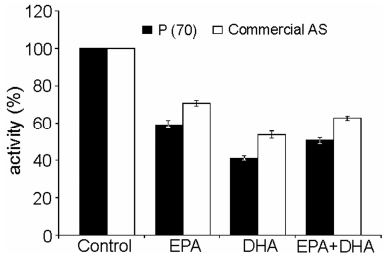

Fig. 2—Effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (8 µg each when used individually) and their mixture (4 µg each) on ASA activity of P70 and of commercial source [Details are described in 'Materials and Methods'. (n = 4). 5 µg enzyme was used in each case]

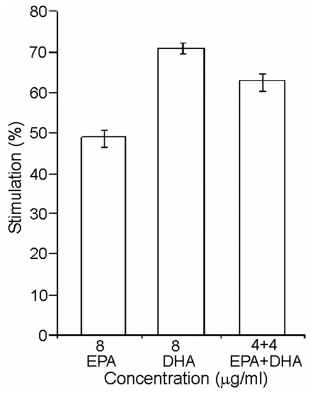

Fig. 3—Effect of EPA and DHA and their mixture on Na^+, K^+-ATPase activity [The ATPase activity was measured in the presence of either EPA or DHA (8 µg/ml each or 4 µg/ml each in the mixture). Percent stimulation of is shown over the control activity of Na^+, K^+-ATPase in absence of EPA or DHA. Other assay conditions as described in 'Materials and Methods'. (n = 3)]

The activity of Na^+, K^+-ATPase is regulated by the content and composition of membrane lipids and their associated unsaturated fatty acids[18]. Thus, we examined the effect of EPA and DHA on ASA and Na^+, K^+-ATPase activities. ASA activity of P70 and commercial source was inhibited by addition of DHA and EPA almost to the same extent and no additive effect was observed when their mixture was used in equal proportion (Fig. 2). Na^+, K^+-ATPase (purified from rat brain) increased to about 49% and 71% in presence of EPA and DHA respectively at a concentration of 8 µg/ml and the additive effect was observed with the mixture (Fig. 3).

Both P70 (containing ASA activity) and commercial AS inhibited the Na^+, K^+-ATPase activity to about 70% and 56% respectively at 8 µg/ml concentration (data not shown). EPA and DHA inhibited ASA activity of P70 and commercial AS, and stimulated the Na^+, K^+-ATPase activity. We

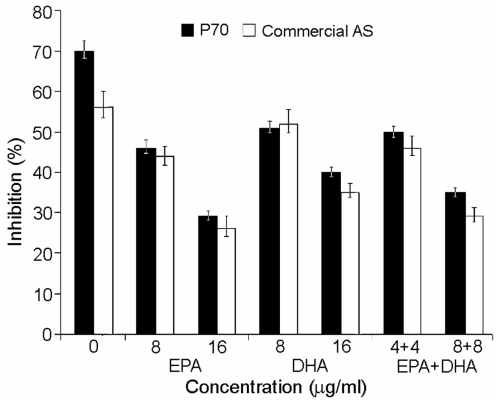

Fig. 4—Inhibition of Na^+, K^+-ATPase activity by P70 and commercial AS in presence of different concentrations of EPA, DHA and their mixture [The standard assay condition was followed as described in "Materials and Methods" (n = 4)]

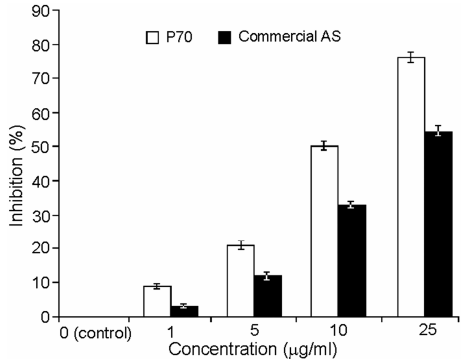

Fig. 5—Effect of anti-P70 antibody on the inhibition of Na^+, K^+-ATPase activity by P70 and commercial AS [The standard assay procedure was followed for the measurement of activity of ATPase as described in 'Materials and Methods']

examined the inhibition of ATPase activity by P70 and commercial ASA in presence of EPA and DHA to study, if the inhibitory effect of sulphatase could be countered by PUFAs. The inhibition of Na^+, K^+-ATPase by P70 and commercial AS was reduced in presence of either EPA or DHA alone and their mixture. The findings suggested that PUFAs reduced the inhibitory effect of AS's (P70 and commercial one), thus decreased the inhibition of Na^+, K^+-ATPase activity (Fig. 4).

The anti-P70 antibody inhibited ASA activity of both P70 and commercial one. Although a concentration-dependent inhibition of AS activity was observed in both the cases, the effect was higher on AS associated with P70 at different concentrations studied, suggesting that P70 was more sensitive to the anti-P70 antibody than the commercial one (Fig. 5)

Western blot analysis (Fig. 6) showed that both P70 and commercial one responded strongly against anti-

Fig. 6—Immunodetection of P70 and commercial AS with the antibody raised against P70 [Lane 1, AS associated with P70 (5 μg); and lane 2, commercial AS (5 μg) probed with rabbit antisera at a dilution of 1:1000 as primary antibody. The commercial one was considered as reference arylsulphatase]

P70 antibody, with P70 showing stronger intensity. This along with the above-described results suggested similarities between ASA activity of P70 and the one from commercial source. It may be mentioned here that although we used rat brain microsomal membranes as source of Na^+, K^+-ATPase, the same effect was observed from other sources also (data not shown).

In conclusion, P70 isolated from goat spermatozoa was found to be a potent inhibitor of Na^+, K^+-ATPase and showed significant ASA activity, which was comparable to the commercial AS obtained from abalone entrails. Thus, P70 was unique in having both regulatory and catalytic activities.

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