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ABSTRACT

Objective: The germ cell/tissue- specific Protein phosphatase, PP1 γ 2, plays a pivotal role in sperm function. Its activity gets reduced during sperm maturation in the epididymis. Inhibition of PP1 γ 2 results in motility initiation and stimulation. The aim of the present study was to determine the biochemical mechanism of PP1 γ 2-sds22 binding in reference to motility status of Swiss albino rat spermatozoa.

Methodology and results: The enzyme from caudal and caput sperm extracts was purified by column chromatography. PP1 γ 2 from caudal spermatozoa was inactive, whereas in caput spermatozoa it was active. The DEAE-cellulose flow through fractions was next passed through an SP-sepharose column. Caudal sperm sds22 and PP1 γ 2 were co-eluted in the gradient fraction. In contrast, caput sperm sds22 and PP1 γ 2 were separated in the flow-through and gradient fractions, respectively. Further purification through a Superose 6 column showed that PP1 γ 2-sds22 complex from caudal sperm was about 80 kDa in size. Caput sperm sds22 and PP1 γ 2 were observed to be eluted at 65 kDa and 39 kDa, respectively. SDS-PAGE of these purified fractions revealed that in caudal sperm, the 80 kDa species is composed of sds22 (43 kDa) and PP1 γ 2 (39 kDa), suggesting an equal ratio complex between these two key proteins. PP1 γ 2 bound to sds22 in this complex was observed to be inactive.

Conclusion and application of findings: The results indicate that dissociation of sds22 from apparently 22 kDa protein is required for its binding and inactivation of PP1 γ 2. Further studies to determine the mechanisms responsible for development of sds22 binding to PP1 γ 2 during epididymal sperm maturation are in progress.

Key words: Phosphatases, signal transduction, sperm maturation, Swiss albino rat, testis.

INTRODUCTION

Studies pertaining to physiology and biochemistry of mammalian spermatozoa have shown that microsomal membrane vesicles isolated from spermatozoa contain Ca²⁺ ATPase, and exhibit Ca²⁺ transport activities that require exogenous Mg²⁺ (Girija *et al.*, 1994; Shivaji *et al.*, 1995, 1998). The enzyme activity is inhibited by calcium channel

inhibitors, for example verapamil and diltiazem, like the well known Ca²⁺ Mg²⁺ATPase. The uptake of calcium is ATP (energy) dependent and the accumulated Ca²⁺ can be completely released by the Ca²⁺ ionophore A23187, suggesting that a significant fraction of the vesicles are oriented inside out (Shivaji *et al.*, 1995, 1998). Besides, a review



(Sharma *et al.*, 2004) shows that current research in India to develop male contraceptive is directed mainly towards: (a) development of anti-spermatogenic agents to suppress sperm production; (b) prevention of sperm maturation; (c) prevention of sperm transport through the vas deferens (or rendering these sperm infertile); and (d) prevention of sperm deposition.

The testis-specific protein phosphatase, PP1 γ 2, is the putative protein phosphatase in spermatozoa (Smith *et al.*, 1996 a, b). High PP1 γ 2 activity leads to low motility, and vice versa (Smith *et al.*, 1996 b). This lowering in PP1 γ 2 activity during sperm maturation is not due to a reduction in the amount of the enzyme but to a down-fall in its catalytic activity. It has been observed that the protein phosphatase inhibitors, okadaic acid and calyculin A, initiate and stimulate motility of epididymal spermatozoa. The enzyme PP1 γ 2 is known to be associated with the regulation of spermatozoa from all mammalian species studied so far (Smith *et al.*, a, b; Vijayaraghavan *et al.*, 1996; Chakrabarti *et al.*, 2007 a, b). At least four different somatic cell serine/threonine phosphatase types, namely, PP1, PP2A, PP2B, and PP2C, are known (Mumby & Walter, 1993; Wera & Hemmings, 1995; Honkanen & Golden, 2002). The four catalytic subunit isoforms of PP1, encoded by three genes, are PP1a, PP1 γ 1, PP1 γ 2, and PP1 δ (Kitagawa *et al.*, 1990; Sasaki *et al.*, 1990; Wera & Hemmings, 1995). PP1 γ 1 and PP1 γ 2 are alternatively spliced isoforms generated from a single gene (Kitagawa *et al.*, 1990; Sasaki *et al.*, 1990; Wera & Hemmings, 1995). While PP1 γ 1 is ubiquitous, PP1 γ 2 is present only in germ cells and spermatozoa (Kitagawa *et al.*, 1990; Varmuza *et al.*, 1999). These two PP1 variants are identical in all respects except that PP1 γ 2 has a unique 21 amino acid carboxy terminus extension. It is noteworthy that the carboxy terminus extension of PP1 γ 2, which is lacking in somatic cell PP1 isoforms, is conserved in all mammalian spermatozoa studied so far, suggesting sperm-specific roles for this amino acid sequence domain.

Disruption of the PP1 γ gene in mice causes sterility in males because of arrest of spermatogenesis at the spermatid stage (Varmuza

et al., 1999). Studies on protein phosphatases in spermatozoa, until now, have been limited (Ahmad *et al.*, 1995; Aimar *et al.*, 2000; Tash *et al.*, 2001; Chakrabarti *et al.*, 2007 a; Han *et al.*, 2007). Based on the observation that protein phosphatases in the reactivation media prevent motility initiation (Han *et al.*, 2007) of invertebrate spermatozoa, it has long been suspected that protein phosphatase regulates flagellar motility. This observation shows that other isoforms of PP1 can compensate for the lack of PP1 γ 2 in somatic cells and for the lack of PP1 γ 2 in germ cells until the final stages of spermatogenesis. Testis-specific PP1 γ 2 is indispensable in the final stages of spermatogenesis.

The earlier studies focused on understanding mechanisms regulating PP1 γ 2 and determination of the basis for the decline in PP1 γ 2 activity during epididymal sperm maturation. Protein phosphatases, in general, are regulated by their binding and targeting proteins (Oliver & Shenolikar, 1998; Cohen, 2002; Thangaraj *et al.*, 2003; Boe-Hansen *et al.*, 2006; Cebesoy *et al.*, 2006; Andrabi, 2007; Angekopoulou *et al.*, 2007; Bengum *et al.*, 2007; Oliva, 2006; Marchesi & Feng, 2007; Wu & Chu, 2008; Cheng *et al.*, 2009). Following identification of PP1 γ 2, we speculated that one or more somatic cell protein regulators of PP1 might be present in spermatozoa. The initial efforts focused on proteins I1 and I2, which are ubiquitous PP1 inhibitors found in all somatic cells (Oliver & Shenolikar, 1998). Surprisingly, sperm do not contain any detectable I1 or I2, suggesting that mechanisms regulating PP1 γ 2 could be unique (unpublished data). Immunoaffinity chromatography was used for the first time to isolate PP1 γ 2 and its associated proteins (Huang *et al.*, 2002). Sds22 is a prototypic member of a family of proteins containing leucine-rich repeats (Kobe & Deisenhofer, 1995). In caudal epididymal spermatozoa, sds22-bound PP1 γ 2 is catalytically inactive (Huang *et al.*, 2002). The co-immunoprecipitation suggested, but did not prove, that sds22 was bound to PP1 γ 2. Stoichiometry of PP1 γ 2 binding to sds22 was not known. It was also necessary to determine why PP1 γ 2 activity is lowering in caudal compared to caput



spermatozoa. Therefore, the present study was undertaken to purify PP1 γ 2 and sds22, by column chromatography, from both caput and caudal

spermatozoa and to understand how sds22 may regulate PP1 γ 2 activity during sperm maturation and motility initiation in Swiss albino rat.

MATERIALS AND METHODS

Preparation of sperm extracts: The Swiss albino rats were procured from the Central Animal House Facility, IFTM Campus, Moradabad. Testes with intact tunica from the mature rats were excised out carefully. Spermatozoa were isolated from caudal and caput epididymis and washed as previously described (Vijayaraghavan *et al.*, 1996; Mishra *et al.*, 2003 a, b, c) in buffer A (10 mM Tris- HCl [pH 7.2] containing 120 mM NaCl, 10 mM KCl, 5 mM MgSO₄). Sperm pellets were suspended in a homogenization buffer (buffer B) (10 mM Tris [pH 7.2] containing 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine- HCl, 1 mM PMSF, 0.01 mM *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone [TPCK], and 5 mM β -mercaptoethanol). The sperm suspension was sonicated with three 5-sec bursts of a sonicator at maximum setting. The sperm sonicate was then centrifuged at 16000 \times *g* for 10 min. The 16000 \times *g* supernatants were supplemented with 10% glycerol and stored at -20°C until further use for column procedures.

Column chromatography: All column procedures were conducted at 4 to 8°C. Total protein in caudal and caput sperm extracts and fractions obtained from column chromatography was measured with Coomassie brilliant blue dye reagent (Bio-Rad laboratories) as described previously (Bradford, 1976). Caudal sperm extract (50 ml prepared from 5 \times 10¹⁰ spermatozoa in buffer B) was passed through a DEAE-cellulose (0.5cm \times 15 cm) column pre-equilibrated with buffer C (buffer B with 0.05 M KCl and additional protease inhibitors-pepstatin A [1 mg/ml], aprotinin [2 mg/ml], and leupeptin [0.5 mg/ml]). The column was washed with 20 ml of buffer C followed by elution with a linear gradient of 0.05–0.7 M KCl in buffer C. Flowthrough and gradient fractions (0.2–0.4 M KCl) containing PP1 γ 2 activity and PP1 γ 2 and/or sds22 immuno-reactivity were pooled separately. The pooled fractions were concentrated to 12.5 ml using Centricon-10 filter (Millipore Corp., Bedford, MA). A SP-sepharose column (5 ml prepacked, Pharmacia) was pre-equilibrated with buffer C. The DEAE-cellulose flow-through fraction was applied to this SP-sepharose column. The column was then washed with 10 ml buffer C followed by elution with a linear gradient of 0.05–0.7 M KCl in buffer C. Fractions containing sds22 and

PP1 γ 2 were pooled, concentrated to 1 ml, and applied in five batches to Superose 6 (24 ml, prepacked high-resolution FPLC, Pharmacia) column pre-equilibrated with buffer C. The elution was performed with buffer C. Immunoreactive fractions of all five batches were pooled, concentrated to 1 ml, and applied to Mono S (1 ml, prepacked high-resolution FPLC, Pharmacia) column. The column was washed with 5 ml buffer C followed by elution with a linear gradient of 0.05–0.7 M KCl in buffer C. Immunoreactive fractions (0.15–0.20 M KCl) containing sds22-PP1 γ 2 complex were pooled, and concentrated to 0.5 ml. For purification of sds22 and PP1 γ 2 from caput epididymal spermatozoa, we followed the same protocol as described above for caudal sperm, except that 50 ml of extract derived from 1 \times 10¹⁰ spermatozoa was used. The final step for purifying caput sds22-PP1 γ 2 complex through a heparin sepharose (1 ml, prepacked Pharmacia) column was similar to the protocol used for Mono S column chromatography. Purified and concentrated caudal sds22-PP1 γ 2 complex and caput sds22 and free PP1 γ 2 fractions were stored at -20°C until further use.

Protein phosphatase assay: Preparation of radiolabeled phosphorylase a, and its use as a substrate for measurement of PP1 activity is based on a protocol described previously (Vijayaraghavan *et al.*, 1996; Mishra *et al.*, 2003 a, b, c). The substrate, aliquots from sperm extracts, and fractions obtained after column chromatography were incubated (in a total volume of 40 μ l) at 30°C with 1 mM Mn²⁺ and with or without inhibitors for 10 min. At the end of this incubation, the reaction was terminated by adding 180 μ l of 20% trichloro acetic acid, after which the tubes were centrifuged for 5 min at 12000 \times *g* at 4°C. The supernatants were analyzed for ³²PO₄ released from phosphorylase a. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of ³²PO₄/ min under conditions of the enzyme assay. This assay is specific for the protein phosphatases PP1 and PP2A (Vijayaraghavan *et al.*, 1996; Mishra *et al.*, 2003 a, b, c).

SDS-polyacrylamide gel electrophoresis: Sperm extracts (25–50 mg) and aliquots from fractions obtained from column chromatography (2–10 mg) were separated by SDS-PAGE through 12% acrylamide slab



gels based on the protocol of Laemmli (1970). One milliliter of soluble extracts (prepared from 10^9 cells) contain approximately 0.5 mg/ml and 1.5 mg/ml protein for caudal and caput sperm, respectively.

Total protein assay: Total protein was measured with Coomassie Brilliant Blue dye reagent (Bio-Rad) as

described previously (Bradford, 1976). Proteins were precipitated with 10% TCA and dissolved in 0.05M NaOH. This precipitation step was necessary because of the interference in the assay by benzimidazole present in the homogenization buffer.

RESULTS

Purification of PP1 γ 2 and sds22 From Caudal epididymal spermatozoa: Following at least three pilot purification runs, 50 ml of sperm extracts prepared from 5×10^{10} Swiss albino rat caudal epididymal

spermatozoa were used to isolate enough PP1 γ 2 and sds22 for biochemical studies. Specific activity of PP1 γ 2 in the pooled extracts was 2.45 U/mg protein. A summary of the purification steps is shown in Table 1.

Table 1: Summary of sds22-PP1 γ 2 purification from caudal epididymal sperm extracts of Swiss albino rats.

Purification step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)
Caudal sperm extract	205	502	2.45
DEAE – cellulose flow through	83	Inactive	ND
DEAE cellulose gradient (0.2-0.4 M KCl) (PP γ 2 without sds22)	76	396	5.21
SP Sepharose gradient (0.13-0.28 M KCl) of DEAE cellulose flow through	35	Inactive	ND
Superose 6 of SP Sepharose gradient	2.21	Inactive	ND
Mono S gradient (0.15-0.20 M KCl)	1.03	Inactive	ND

Data are purification of three batches of 50 ml each of caudal sperm extract. The total number of cells corresponding to 205 mg protein is 1.5×10^{11} . Activity of PP1 γ 2 in caudal sperm extracts and fractions obtained after column procedures was measured in the presence of 1 mM Mn^{2+} .

A portion of the enzyme was found in the flow-through fraction, and the rest was absorbed in the column. In addition to PP1 γ 2, the DEAE-cellulose flowthrough fraction also contained immunoreactive sds22 (data not shown). The enzyme in the DEAE-cellulose flow-through fraction was inactive whereas that released from the column by a salt gradient (0.20-0.40 M KCl) showed a specific activity of 5.21 U/mg protein.

This manuscript's focus is on the association of PP1 γ 2 to sds22 and details of purification of PP1 γ 2 released from the DEAE-cellulose column are not presented. Here PP1 γ 2 and sds22 were bound in the column. All the bound PP1 γ 2 and sds22 were released by a salt gradient between 0.13–0.28 M KCl. The occurrence of sds22 and PP1 γ 2 in the DEAE-cellulose and SP-sepharose column fractions was assessed by the PP1 γ 2 assay showing the inactivation of these fractions (Table 1). After concentrating the SP-sepharose salt gradient fractions containing both

PP1 γ 2 and sds22 and passing them through a Superose 6 column, they were co-eluted as an 80-kDa species in fractions 27, 28, and 29 (Fig. 1). Co-elution of sds22 and PP1 γ 2 through these three columns, DEAE-cellulose, SP-sepharose, and Superose 6, suggested that they could be complexed.

To confirm their association, we further purified sds22 and PP1 γ 2 containing fractions through a Mono S column, where they were absorbed on the Mono S column and co-eluted in a 0.15–0.20 M KCl gradient. These fractions containing PP1 γ 2 and sds22 were pooled, concentrated and analyzed by SDS-PAGE. Coomassie blue and silver staining following SDS-PAGE (Fig. 2) showed that the complex eluting from Mono S column contains two major protein bands at 43 and 39 kDa. All column fractions containing sds22 and PP1 γ 2 had virtually no protein phosphatase activity (Table 1).



Purification of PP1 γ 2 and sds22 from Caput Epididymal Spermatozoa of Swiss Albino Rat: Subsequently, we subjected caput sperm extracts to essentially the same purification scheme used for caudal sperm extracts. Caput sperm extracts have

higher PP1 γ 2-specific activity: 12.3 U/mg protein, compared to 2.45 U/mg protein in caudal sperm. A summary of the purification of sds22 and PP1 γ 2 from Swiss albino rat-caput sperm extracts is highlighted in Table 2.

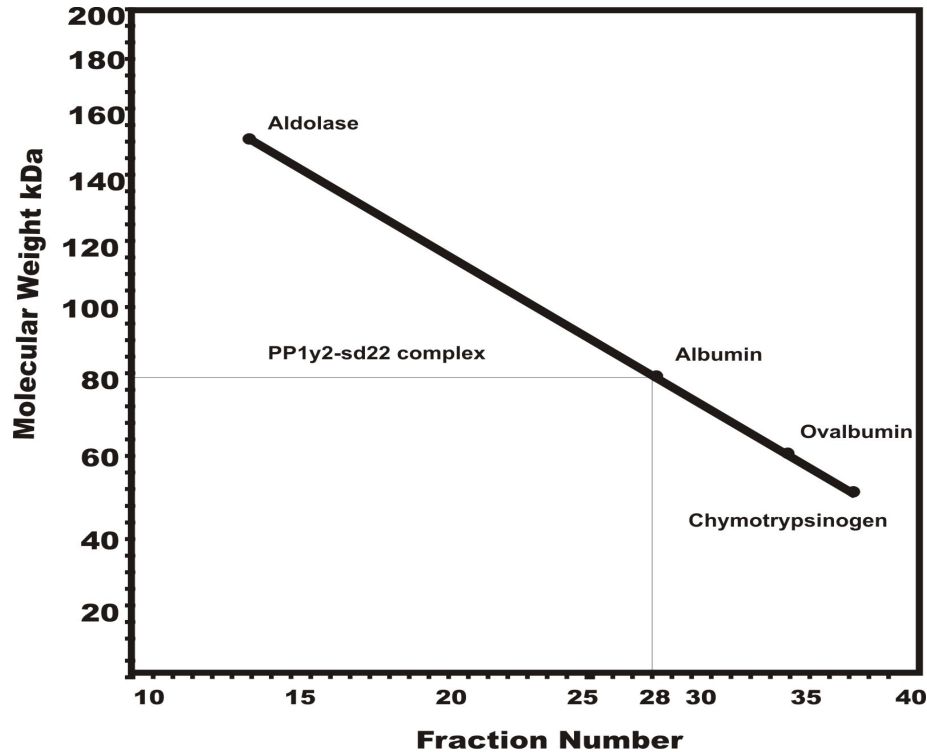


Figure 1: Molecular weight determination of the caudal sperm PP1 γ 2- sds22 complex. Elution profile of PP1 γ 2-sds22 complex from caudal sperm extracts in Superose 6. PP1 γ 2-sds22 complex eluted through Superose 6 at 80 kDa.

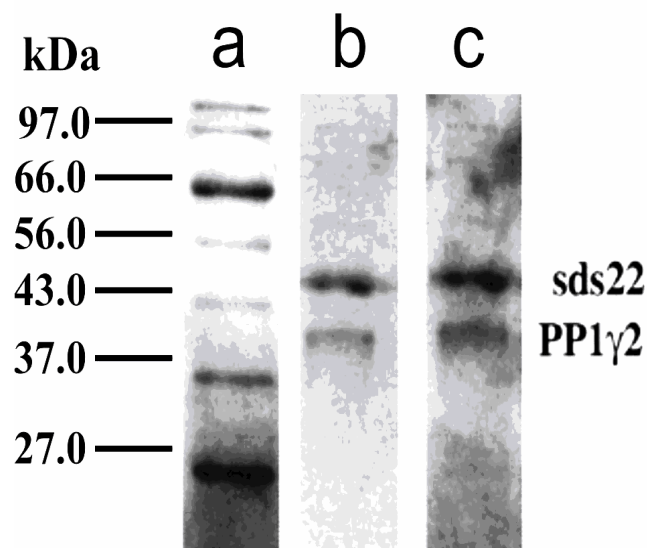


Figure 2: Caudal sperm PP1 γ 2 is bound to protein sds22. **Lane A** shows protein molecular weight markers. Coomassie blue (**Lane B**) and silver stained gels (**Lane C**) showing PP1 γ 2 and sds22 after SDS-PAGE of purified PP1 γ 2-sds22 complex obtained after Mono S column chromatography (2 mg). Silver staining was performed on the same gel following Coomassie blue.

Table 2. Summary of sds22 and PP1 γ 2 purification from caput epididymal sperm extracts of Swiss albino rats.

Purification step	Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery %	Purification fold
Caput Sperm Extract	68.5	843.92	12.32	100	1
DEAE cellulose flow through (sds22 +PP1 γ 2)	31.7	65.71	25.81	7.8	2.1
DEAE cellulose gradient (0.2-0.3 M KCl) (PP1 γ 2 without sds22)	35.3	616	17.45	73	1.4
SP Sepharose flow through of DEAE cellulose flow through (free sds22)	17.6	Inactive	ND	ND	ND
Superose 6 (sds22)	1.6	Inactive	ND	ND	ND
Heparin Sepharose flow through (sds22)	0.78	Inactive	ND	ND	ND
SP Sepharose gradient (0.4-0.5 M KCl of DEAE cellulose flow through) (free PP1 γ 2)	6.6	241	36.75	29	3.0
Superose 6 (free PP1 γ 2)	0.26	217	835	26	68

NB: Data are purification of three batches of 50 ml each of caput sperm extract. The total number of cells corresponding to 68.5 mg protein is 3×10^{10} . Activity of PP γ 2 in caput sperm extracts and fractions obtained after column procedures was measured in the presence of 1 mM Mn²⁺.

Similar to caudal sperm extracts, PP1 γ 2 in caput sperm extracts also separated into flow-through and gradient fractions on DEAE-cellulose. In addition to PP1 γ 2, the DEAE -cellulose flow-through fraction also contained immunoreactive sds22 (data not shown). However, unlike caudal sperm (Table 1), PP1 γ 2 in the DEAE-cellulose flow-through fraction of caput sperm extracts was catalytically active: 25.8 U/mg protein (Table 2). The enzyme released by a salt gradient (0.20–0.30 M KCl) from the DEAE-cellulose column had a specific activity of 17.5 U/mg protein.

After passing the DEAE-cellulose flow-through fraction through an SP-sepharose column, sds22 was found in the flow through fraction, but PP1 γ 2 was absorbed by the matrix and was eluted by a salt gradient (0.40–0.50 M KCl). Specific activity of enzyme eluting from SP-sepharose was 36.8 U/mg protein (Table 2). The SP-sepharose column fractions containing sds22 and PP1 γ 2 were concentrated and passed through a Superose 6 column. Caput sperm sds22 eluted as a 65-kDa protein, fractions 30, 31 and 32 (Fig. 3). This 65-kDa protein was further passed

through a heparin sepharose column, in which immunoreactive sds22 was found in the flow-through fraction (data not shown). SDS-PAGE followed by Coomassie blue and silver staining reflects that heparin Sepharose-purified 65 kDa species is composed of two major protein bands at 43 kDa and 22 kDa (p22). The SP-sepharose fractions containing PP1 γ 2 eluted through the Superose 6 column at about 39 kDa: a molecular size in close correspondence to the calculated size based on its amino acid sequence (39 kDa) (data not shown). Identification of the purified PP1 γ 2 and sds22 is underway to confirm through Western blot analysis based on the preliminary experiments carried out using ELISA.

Caput Sperm sds22-p22 Complex is Observed Not to Bind to PP1 γ 2: Caudal but not caput sperm sds22 is bound to PP1 γ 2. A logical question is whether the lack of sds22-PP1 γ 2 binding is due to some modification in PP1 γ 2 in caput spermatozoa. If this were the case, purified caput sperm sds22 should bind to recombinant PP1 γ 2 extracts expressed in bacteria. It

was investigated whether the purified caput sperm sds22-p22 complex can inhibit recombinant PP1 γ 2 *in vitro*. Variable concentrations of heparin sepharose-purified sds22-p22 complex (0 to 2 mg) were pre-incubated with PP1 γ 2 (Table 2) followed by measurement of protein phosphatase activity. Catalytic activity of PP1 γ 2 activity was not significantly inhibited at any concentration of sds22 (Fig. 4). Calyculin A (5

nM), a well known inhibitor of PP1, does inhibit catalytic activity of the recombinant enzyme (Fig. 4), reflecting that the recombinant enzyme is capable of binding to these inhibitors. Identical results to those shown in Figure 4 were also obtained when recombinant PP1 α or PP1 γ 2 purified from caput sperm extracts (Superose 6 eluate; Fig. 3) instead of recombinant PP1 γ 2 was used (data not shown).

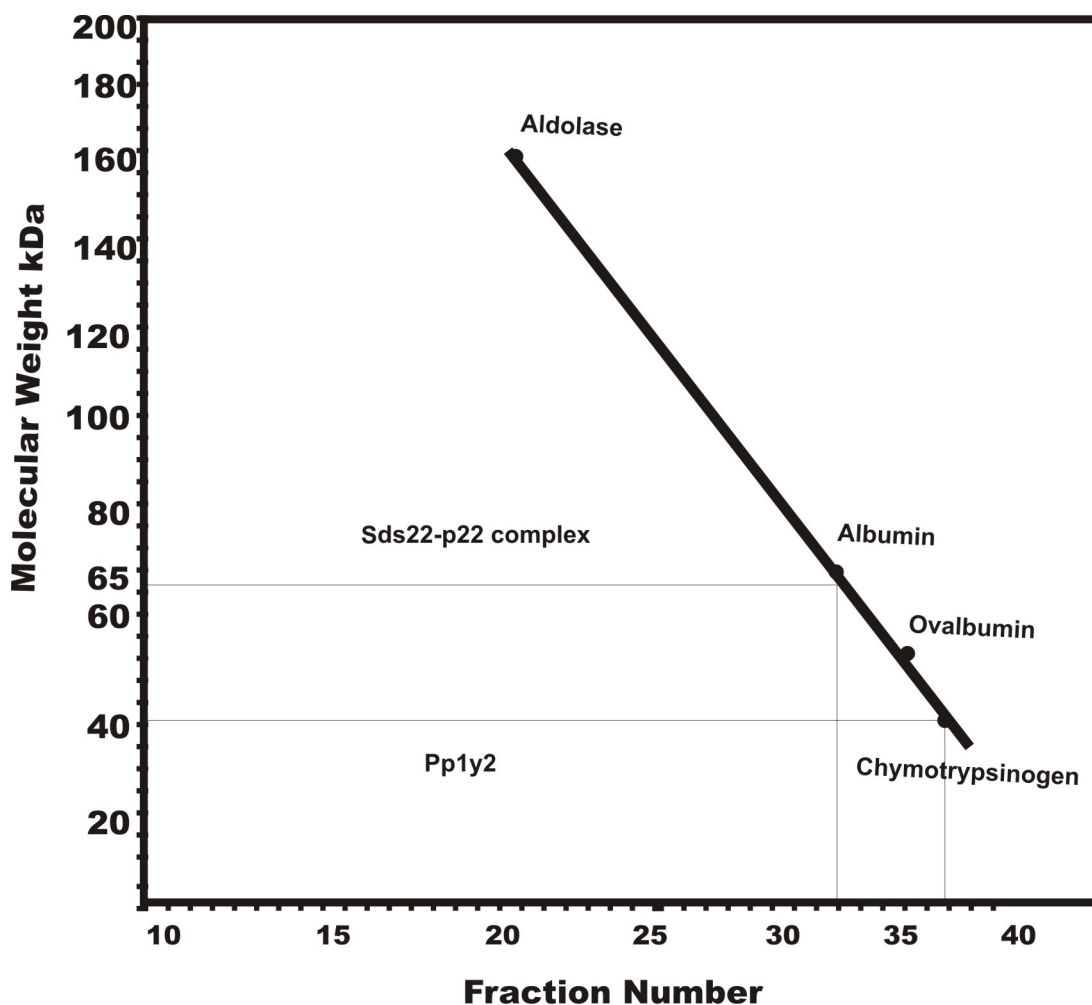


Figure 3: Molecular weight determination of the caput sperm PP1 γ 2 and sds22 of Swiss albino rats. Elution profile of sds22-p22 complex and PP1 γ 2 from caput sperm extracts in Superose 6. PP1 γ 2 and sds22-p22 complex eluted through Superose 6 at 39 kDa and 65 kDa, respectively.

DISCUSSION

It is well known that PP1 isoforms are regulated by several ubiquitous and cell-specific regulatory and targeting proteins in case of somatic cells (Oliver & Shenolikar, 1998). Germ cell-specific protein phosphatase PP1 γ 2 is the putative serine/threonine phosphatase in spermatozoa (Smith *et al.*, 1996 a, b; Vijayaraghavan *et al.*, 1996). The activity of this

enzyme is inversely correlated with motility: low activity in vigorously motile caudal spermatozoa and high activity in immotile caput spermatozoa (Smith *et al.*, 1996 b). Inhibition of the enzyme with okadaic acid and calyculin A causes motility initiation and stimulation (Smith *et al.*, 1996 a, b; Vijayaraghavan *et al.*, 1996). Our focus was to understand how PP1 γ 2 is regulated

and how the activity of the enzyme declines during epididymal sperm maturation. Antibodies to PP1 γ 2 co-precipitate sds22 from sperm extracts suggesting that sds22 is bound to PP1 γ 2 (Huang *et al.*, 2002). One of the objectives of this study was to confirm this observation by column purification techniques and determine stoichiometry of the PP1 γ 2-sds22 complex. Caudal sperm sds22 co-purifies with PP1 γ 2 through four different columns: DEAE-cellulose, SP sepharose, Superose 6, and Mono S. The fact that sds22 and

PP1 γ 2 co-purify in column chromatography and immunoprecipitation (Huang *et al.*, 2002) strongly supports the conclusion that sds22 is complexed to PP1 γ 2. The apparent molecular weight of the sds22 and PP1 γ 2 complex, determined by Superose 6 column chromatography, is 80 kDa. This is in close correspondence with a molecular weight of 82 kDa, for an equal ratio PP1 γ 2-sds22 complex, assessed as per their amino acid profiles.

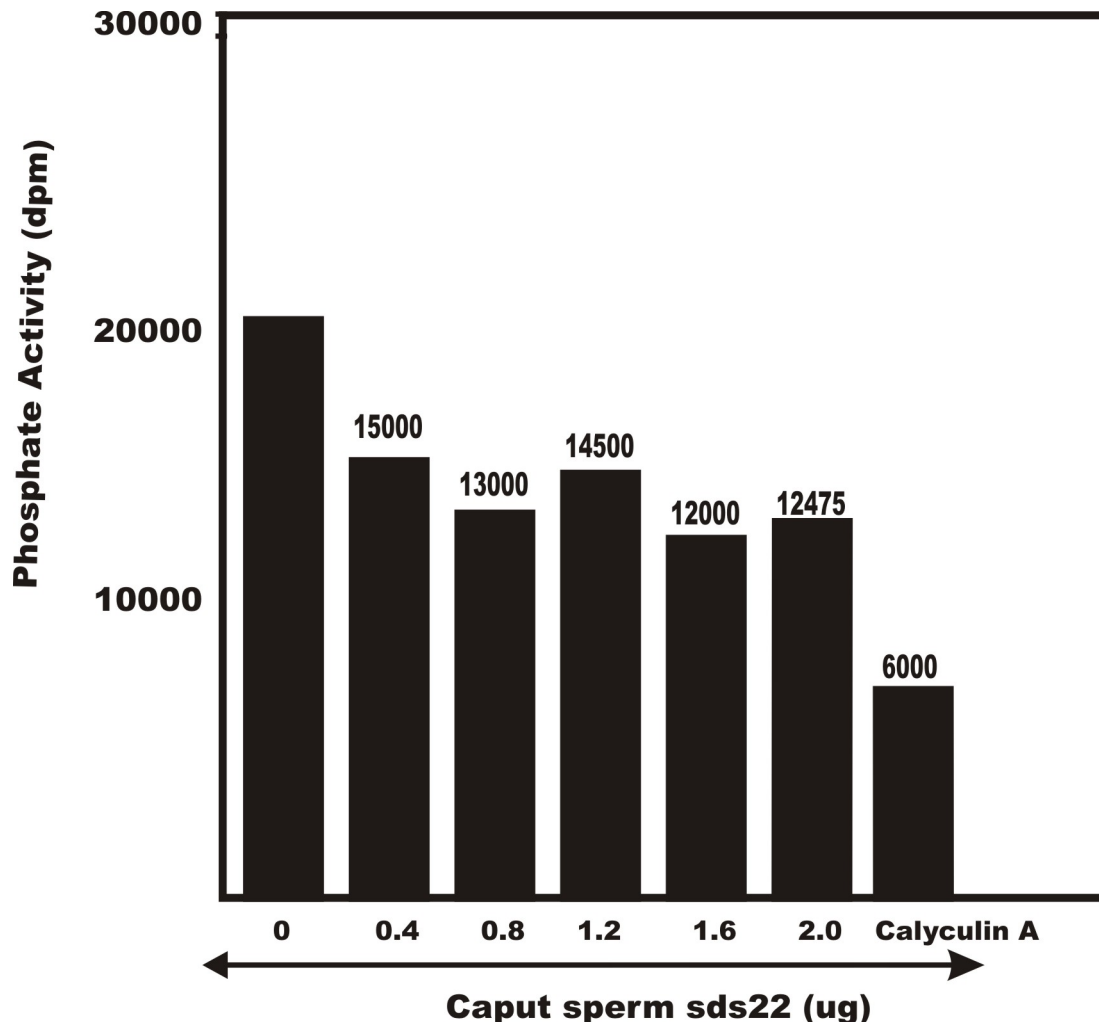


Figure 4: Effect of caput sperm sds22-p22 complex on catalytic activity of recombinant PP1 γ 2. The indicated amounts of sds22-p22 complex purified from caput sperm extracts, 5 nM calyculin A were added to recombinant PP1 γ 2 (100 mU) and incubated at 30°C for 15 minutes. After this incubation, the phosphatase assay was initiated with phosphorylase.

Our data do not rule out the possibility that some of the caudal sperm sds22 may exist as a homodimer (86 kDa). It is, however, unlikely that this sds22 will co-elute with the sds22-PP1 γ 2 complex through the four

different column matrices. Furthermore, free sds22 (i.e., sds22 not bound to PP1 γ 2) exists as a 65-kDa species in caput spermatozoa (Fig. 3). It is noteworthy that immunoreactive sds22 is not detected in any other

column fraction except those also containing PP1 γ 2 (Table 1) suggesting that all of the sds22 present in caudal sperm extracts is bound to PP1 γ 2 (i.e., sds22 in sperm extracts is a PP1 γ 2 regulatory protein). In accordance with earlier observations made with crude caudal sperm extracts (Huang *et al.*, 2002), we also determined that column-purified sds22-PP1 γ 2 complex does not also bind to microcystin (data not shown). Microcystin can bind to PP1 and PP2A catalytic subunits alone or when the catalytic subunits are associated with a variety of their regulatory proteins (Meek *et al.*, 1999). In particular, the sds22 complex with the somatic cell PP1 isoform, PP1 α , can bind to microcystin (Tran *et al.*, 2002). The inability of the sperm sds22-PP1 γ 2 complex to bind to microcystin suggests that sds22 masks the microcystin-binding site on PP1 γ 2. Purified PP1 γ 2-sds22 complex and the complex present in the different column fractions are virtually devoid of catalytic activity (Table 1).

The sds22-PP1 γ 2 complex isolated by immunoaffinity chromatography from crude sperm extracts was also inactive (Huang *et al.*, 2002). Thus sds22 is a PP1 γ 2 inhibitor in spermatozoa. It is noteworthy that spermatozoa do not contain the ubiquitous somatic cell PP1 inhibitors I1 and I2 (our unpublished data). Data presented in this report provide strong evidence that in motile caudal spermatozoa PP1 γ 2, present as an equal ratio complex with sds22, is catalytically inactive.

The next objective of this study was to examine whether sds22 in caput epididymal spermatozoa is also bound to PP1 γ 2. Data presented in Table 2 and Figure 3 clearly reflect that caput sperm sds22 is not bound to PP1 γ 2. Unlike caudal sperm extracts, PP1 γ 2 from caput spermatozoa in the DEAE cellulose flow-through fraction and SP-sepharose gradient fractions is catalytically active. The specific activity of caput sperm PP1 γ 2, not bound to sds22, following Superose 6 column purification, is 567 U/mg protein. The enzyme at this stage of purification (68-fold purification, compared to crude extracts, Table 2) is not expected to be homogenous. Our principal motto here was not purification of the enzyme to homogeneity but to assess whether the enzyme was bound to sds22 or some other regulatory protein. The apparent molecular weight of the active subunit of the enzyme eluting through the Superose 6 column is 39 kDa in correspondence with the calculated molecular weight (39 kDa) based on its amino acid profile. In caput sperm, active PP1 γ 2 is not bound to sds22 or any other regulatory protein. One of the reasons for the high

protein phosphatase activity in caput extracts is due to this 39-kDa free catalytic subunit form of PP1 γ 2. Our studies also show that a portion of PP1 γ 2 in both caput and caudal sperm extracts is bound to DEAE-cellulose. Specific activities of PP1 γ 2 released from DEAE-cellulose are 17.5 and 5.2 U/mg protein in caput and caudal sperm, respectively (Tables 1 and 2). One of the important questions emerging from this study is why sds22 from caput spermatozoa is not bound to PP1 γ 2. Caput sperm sds22 elutes as a 65-kDa species through the Superose 6 column (Fig. 3). This 65-kDa species consists of sds22 (43 kDa) and another 22-kDa protein (p22) (data not shown). This suggests that sds22 may exist as a complex with p22 in caput spermatozoa. It is therefore possible that p22, bound to sds22, prevents sds22 from binding to PP1 γ 2. Caput sperm sds22 complexed to p22 is also unable to inhibit recombinant PP1 γ 2 *in vitro* (Fig. 4), presumably because it cannot bind to it. Partially purified caput sperm PP1 γ 2 behaves like recombinant PP1 γ 2 with respect to inhibition by calyculin A and binding to microcystin. Thus, the reason for inability of sds22 to bind to PP1 γ 2 probably is not due to a modification of the PP1 γ 2 subunit but most likely is due to the fact that p22 bound to sds22 prevents formation of the sds22-PP1 γ 2 complex. Dissociation of the sds22-p22 complex and sds22-PP1 γ 2 complex formation may be caused by phosphorylation or some other chemical modification of sds22 or p22. Alternatively, p22 may be proteolyzed during epididymal sperm maturation (i.e., p22 may be absent in caudal spermatozoa). Molecular characterization of p22, development of antibodies against p22, and determination of the status of p22 in caudal spermatozoa will help us to explore mechanisms underlying sds22 dissociation from p22 followed by binding to PP1 γ 2 during epididymal sperm development.

Conclusively our data demonstrate that purified caput sds22 is free of PP1 γ 2. It could be argued that p22 may not be bound to sds22 but is a protein contaminant co-eluting with sds22 in the Superose 6 column (Fig. 3). Other mechanisms may be responsible for the development of sds22 binding to PP1 γ 2. It may be possible that sds22 phosphorylation may be necessary for its binding to PP1 γ 2 (i.e., sds22 may be phosphorylated in caudal but not caput spermatozoa). Insights into these questions might have been provided if we were able to immunoprecipitate sds22 from sperm extracts. Whatever the reason for the lack of binding of sds22 to PP1 γ 2 may be, our data clearly show that all of sds22 in caudal sperm extracts



is bound to PP1 γ 2, whereas in caput sperm extracts, sds22 is free of any detectable PP1 γ 2. A consequence of this difference is that a proportion of caput sperm PP1 γ 2 is in its free (39 kDa) catalytically active form, with a specific activity of 567 U/mg protein after partial purification through Superose 6 column (Table 2). Thus, one of the reasons for the higher PP1 γ 2 activity in caput than in caudal spermatozoa is that PP1 γ 2 is not bound to sds22. Further, the studies described herein clearly indicate that a fraction of PP1 γ 2 in motile caudal epididymal spermatozoa is inactive and present as a heterodimer with sds22. In immotile caput spermatozoa, sds22 is not bound to PP1 γ 2 but is either free or bound to a 22-kDa protein. Therefore, a portion of caput sperm PP1 γ 2 is in its catalytically active and free form. It is therefore very likely that the reason for the higher PP1 γ 2 activity in caput, compared to caudal spermatozoa might be due to the inability of sds22 to bind and inactivate PP1 γ 2. We hypothesize that the change in binding partners of sds22 from p22 to PP1 γ 2

and the development of the binding capacity of sds22 for PP1 γ 2 are key biochemical events responsible for the decline in protein phosphatase activity during epididymal sperm maturation and motility initiation.

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