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Biochemical and Biophysical Research Communications 354 (2007) 265–270
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The N-terminal of a heparin-binding sperm membrane mitogen possess lectin-like sequence ☆

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Received 24 December 2006
Available online 3 January 2007

Abstract

Glycosaminoglycans like heparin and heparin sulfate in follicular fluid induce changes in the intracellular environment during the spermatozoal functional maturation. We previously reported the isolation, purification and partial characterization of a heparin binding sperm membrane protein (HBSM). In the present study, the amino acids analysis provided evidence of a single sequence, which suggest the homogeneity of the purified HBSM. Fourteen amino acids—^{1}**A D T I V A V E L D T Y P N**^{14}—correspond to the amino terminal sequence of Concanavalin A (Con A) and contain 45.2% carbohydrate by weight. HBSM possess mitogenic property on lymphocytes with comparable magnitude to the well-known mitogen; Con A, inducing 83% radiolabel thymidine incorporation in growing lymphocytes. Unlike Con A, there was no agglutination of cell by HBSM upto 5 ng/ml concentration. Interestingly, we found that heparin and chondroitin sulfate-conjugated HBSM inhibit the proliferative activity. Similar effect was also found with an in-house isolate sulfated glycans; G-I (28% sulfate). In contrast, there was no inhibition by the desulfated form; G-ID. Altogether, our data suggest that the mechanism of cell proliferative pathway may be different for HBSM and Con A.

Keywords: Amino-terminal sequence; Cell agglutination; Cell proliferation; Con A; Heparin; Heparin-binding sperm membrane mitogen

Membrane fusion is an important process in eukaryotic cells. The sperm-egg interaction at fertilization is mediated by specific complementary surface molecules like a set of protein, glycoprotein, and carbohydrate present on both the sperm and egg membrane. Sperm membrane proteins also play important role on spermatozoal maturation in the epididymal microenvironment, during which, the cell undergoes a series of morphological, physiological, and biochemical modifications to acquire the successful fertilizing ability. Physicochemical and binding studies reveal that several sperm membrane proteins involved in the cell maturation and zona pellucida interaction possesses strong affinity to heparin [1–4]. Sperm from bull with higher fertility exhibited greater affinities to heparin and bulls subjected to summer heat stresses showed sperm with low affinities to heparin and low fertility [5]. Heparin-binding protein/glycoprotein isolated from various tissues and cells possesses multifunctional property and is particularly important in the biological activities. Fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) belongs to the heparin-binding growth factor family (HBGF) and are well-known endothelial mitogen [6–9]. Heparin binding proteins are also crucial for normal hearth functioning, null mice ($HB^{del/del}$) were reported to have a high mortality rate and the few survived develop severe heart failure [10,11]. Heparin-binding growth-associated molecule (HB-GAM) is a newly discovered HSPG-bound factor and plays important roles in autocrine or paracrine type regulation of growth and differentiation [12]. Insulin-like growth factor-binding protein-3 (IGFBP-3) contains high-affinity heparin-binding site and that heparin-binding blocks the multimerization of IGFBP-3 [13], which is important for regulating the actions of insulin-like growth

☆ Financial Support: Research fellowship provided to Visesato Mor by University Grants Commission, New Delhi, India.
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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.196

factors (IGFs). The angiogenic factor, heparin-affinity regulatory peptide (HARP) is also reported to induce proliferation of human peripheral blood mononuclear cells [14].

We have isolated a heparin-binding sperm membrane protein (HBSM) from goat cauda epididymal spermatozoa and suggested the association of HBSM with sperm cell maturation [15]. The present study describes the amino terminal sequencing of HBSM, which is identical with N-terminal sequence of Con A. To further explore the biological activities of HBSM, we examined cell agglutination and mitogenic property and reported that HBSM lack the cell agglutinating activity but induce cell proliferation with magnitude similar to the T-cell mitogen, Con A.

Materials and methods

Reagents and chemicals. Concanavalin A (Con A), heparin, phytohemagglutinin (PHA), Ponceau S, PVDF membrane, RPMI-1640, and Schiff's reagent were purchased from Sigma (St. Louis, MO). [^{3}H]Thymidine and penicillin–streptomycin were obtained from GIBCO (Life Technologies, Gaithersburg, MD). Chondroitin sulfate C from Calbiochem (EMD Biosciences Inc., Darmstadt, Germany). Orcinol is from SRL (SISCO Research Laboratories Pvt. Ltd, Mumbai, India). G-I and G-ID were gifted by Dr. A.K. Sen Sr. [16].

All other reagents are of the highest analytical grade.

Samples. Goat epididymal tissues and sheep red blood cells were collected from local slaughterhouse and Sprague–Dawley rats were breed in-house.

Glycoprotein staining. The samples were resolved by 10% SDS–polyacrylamide gel and fixed in 50% (v/v) methanol containing 7% (v/v) acetic acid for 24 h followed by washing two times with distilled water for 1 min each. The fixed gel was oxidized in a solution of 5% (v/v) acetic acid containing 1% (w/v) periodic acid for 1 h. After washing two times, the proteins were reduced with 0.5% (w/v) sodium metabisulfite in 5% (v/v) acetic acid for 20 min. The gel was washed with distilled water for two times and kept overnight in Schiff's reagent for detection of glycoprotein.

Sugar estimation. Sugar estimation was performed with orcinol–sulfuric acid methods. Orcinol reagent [0.2% (w/v) orcinol in 70% (v/v) H_2So_4 in water] was prepared and kept cool. Standard samples (glucose/sucrose) were prepared with different concentration; 0.5, 1.0, 1.5, and 2 μg from 1 mg/ml stock solution. Reaction was started by adding 1.5 ml orcinol reagent to 0.5 ml of diluted samples in test tubes and kept in boiling water bath for 20 min. After cooling, the optical density was recorded at 450 nm against orcinol reagent–water blank.

Amino terminal sequencing. Heparin-binding sperm membrane mitogenic protein (HBSM) was purified from heparin–agarose affinity column [15]. The protein sample was dialyzed against 10 mM phosphate buffer (pH 7.0) containing 250 mM NaCl with four changes for 6 h each. Concentration of the dialyzed sample was equivalent to 1.5 at OD_{280}. The sample was subjected to Waters 510 HPLC system equipped with 7.5×300 mm TSK-gel G3000SW column (LKB, Amersham). Separation was performed at 2 ml/min flow rate and the absorbance of eluted fractions was measured at 280 nm. The peak fractions were concentrated and ≈15 μg protein (following SDS–PAGE) was electro-transferred to 0.4 μm pore size PVDF membrane (Sigma) using 10 mM CAPS transferring buffer (pH 11) containing 10% (v/v) methanol at 100 mA for 6 h. The membrane was treated with 70% (v/v) methanol for 5 s and rinsed several times with milli-Q water. The membrane was stained with 0.2% (w/v) Ponceau S to locate the protein band then completely destined with milli-Q water. The single protein band on PVDF membrane after drying was excised and used in sequence analysis. N-terminal amino acid sequence was determined using Applied Biosystem Procise sequencer with smooth degree 9 interpolated baselines. Sequence homology Blast search was performed using NCBI web link Swissprot database (www.ncbi.nih.gov/BLAST).

Cell culture. Spleen cells from Sprague–Dawley rats (250–280 gm body weight) were prepared by rolling the tissue between two frosted ended slide and collected in RPMI-1640 medium (pH 7.2) containing 1 ml/100 ml penicillin–streptomycin. Cell count was carried using haemocytometer. The washed cells (2×10^5) were cultured in RPMI-1640 medium supplemented with 10% FCS at 37 °C in humidified atmosphere with 5% CO_2 in 96-well culture plates for 72 h. Following this incubation period, 1 μCi/well [^{3}H]thymidine was added to each well and culturing was continued for the next 18 h. The test cultures contained either Con A, PHA or different concentration of HBSM. The cells were harvested to glass fibre filter paper using cell harvester and digested with Cocktail "O" in scintillation vial. Radioactive thymidine uptake by the cultured cells was measured using liquid scintillation counter [Packard Tri-carb 31000TR].

Agglutination test. Sheep red blood cells (SRBC) collected in ALSEVERS' solution [2.05% (w/v) dextrose, 0.8% (w/v) $Na_3C_6H_5O_2{\cdot}2H_2O$, 0.0558% (w/v) $C_6H_8O_7{\cdot}H_2O$, and 0.42% (w/v) NaCl, pH 6.1] were washed with 50 mM Tris-buffered saline (TBS) (pH 7.2). The SRBC pellet was made to 10% (pack volume) with TBS. Two percent SRBC suspension was incubated with the agglutination mixture consisting of; HBSM or Con A, 1 mM each of $MnCl_2$ and $CaCl_2$ in 96-well round bottom ELISA plate. The final volume was made to 200 μl/well with 50 mM TBS (pH 7.2). Leukocyte was prepared by incubating the whole blood (3 ml) in 9 ml erythrocytes lysing buffer (0.155 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) at room temperature for 10 min with gentle mixing followed by centrifugation for 5 min at 1200*g*. The whitish pellet was washed three times with 3 ml erythrocyte lysing buffer. The pellet was made to 10% (pack volume) with 50 mM TBS (pH 7.4). Five percent of leukocytes suspension was used for the leukocyte agglutination assay. The agglutination was observed after 3–4 h of incubation at 32 °C. Photograph was taken using an Olympus digital camera.

Statistical analysis. Significant difference in lymphocytes proliferation between HBSM and mitogen/sulfated compounds conjugate was determined by Student's *t* test. Overall significance was determined by analysis of variance (ANOVA) using Sigmastat 3 (SPSS Inc., Chicago). $P < 0.05$ was considered significant.

Result

Carbohydrate estimation

The heparin-binding sperm membrane mitogen shows positive staining for periodic acid Schiff's reagent indicating a glycoprotein in nature. Fig. 1A shows the positive staining for periodic acid Schiff's reagent. Sperm membrane protein extract in lane 1, purified HBSM in lane 2, and glycoprotein (PHA and LPS) as control in lanes 3 and 4. The total sugar content of both native and denatured HBSM was determined by orcinol–H_2SO_4 method. The result shows that native HBSM contains 45.2% carbohydrate by weight while the denatured sample contains 39% (Fig. 1B). Denaturation of HBSM showed no significant different in the carbohydrate content. However, antibody rose against the native HBSM did not recognize the denatured protein (data not shown).

Amino terminal analysis

HPLC fraction (peak II, Fig. 2A) was electro-transferred to PVDF membrane for sequence analysis. The amino terminal residues read as Ala-Asp-Thr-IIe-Val-Ala-Val-Glu-Leu-Asp-Thr-Tyr-Pro-Asn detected from 14 cycles. Table 1 presents the single letter code "ADTIVA-

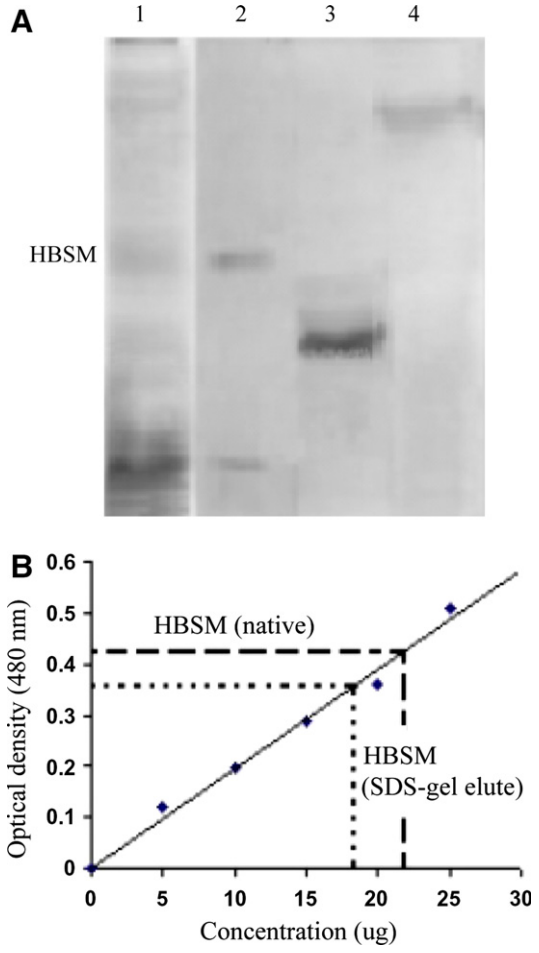

Fig. 1. Periodic acid Schiff's (PAS) staining and total sugar estimation of HBSM. Samples resolved on 10% SDS–PAGE and stained with PAS (A). Lane 1, CHAPS solubilized membrane supernatant; lane 2, purified HBSM; lane 3, PHA-M and lane 4, LPS. (B) Standard plot was drawn using glucose and the sugar content of both native and denatured HBSM was determined using orcinol–sulfuric acid reagent. Optical density was recorded at 490 nm.

VELDTYPN'' which corresponds with the N-terminal residue 1–14 of Concanavalin A (Con A) and Concanavalin Br (Con Br). The sequence revealed presence of several metal-binding sites such as ^{8}Glu; manganese, ^{12}Tyr and ^{14}Asn; calcium, and ^{10}Asp have calcium and manganese binding site. These metal binding sites are important for cell agglutination through the recognition and binding of saccharides [17]. The sequence information led us to consider whether HBSM posses the agglutinating property. Moreover, the sperm plasma membrane proteins are also known to have strong agglutinating property and this is imperative for the recognition and fusion with the zona pellucida during fertilization

Cell agglutination

Erythrocyte agglutination has been considered for long time as a functional test for Con A. Based on the amino terminal sequence identity with Con A, studies were conducted on hemagglutination by HBSM as a functional test. Fig. 3A shows that SRBC was agglutinated at the low concentration of Con A (0.25 ng/ml), whereas, no agglutination was observed with HBSM at the same concentration. No agglutination was observed even at 5 ng/ml concentration. We also examined agglutination of other cells such as leukocyte and yeast by HBSM. In case of leukocyte, no agglutination was noticed with increasing concentration (0.05–5 ng/ml) of HBSM (Fig. 3B). Since PHA also belongs to the lectin mitogen family, we examined the leukocytes agglutination using PHA as positive test.

Con A is known to agglutinate yeast cells. However, there was not agglutination caused by HBSM on the yeast strain; *Saccharomyces cerevisiae* and *Candida albicans*. These results suggest that unlike Con A, HBSM doesn't posses agglutinating property.

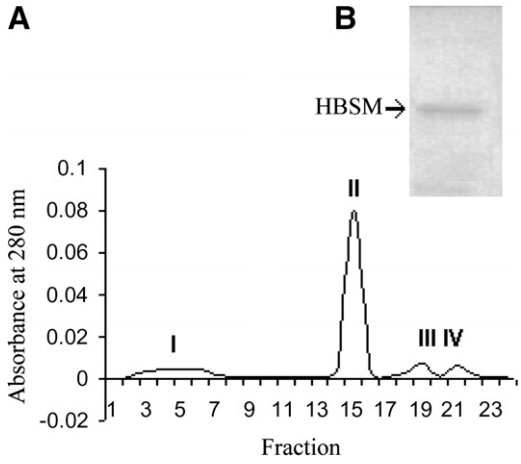

Fig. 2. HPLC purification of HBSM and Ponceau staining on PVDF membrane. Sample eluted from heparin affinity chromatography was subjected to HPLC; the fractions were measured at 280 nm (A). The major peak fraction (II) was concentrated and electro transfer to PVDF membrane following SDS–PAGE. The transferred HBSM was observed on membrane by Ponceau S staining (B).

Table 1
N-terminal amino acid of heparin-binding sperm membrane mitogen and alignment with Con A and Con Br

^{1}Ala-Asp-Thr-IIe-Val-Ala-Val-Glu-Leu-Asp-Thr-Tyr-Pro-Asn^{14}	
Sequence alignment of HBSM with Con A and Con Br	
HBSM	^{1}**ADTIVAVELDTYPN**^{14}
Con A	^{1}**ADTIVAVELDTYPN**^{14} . ^{190}**FDATFTFLIK**^{201}
Con Br	^{1}**ADTIVAVELDTYPN**^{14} . ^{190}**FEATFTFLIK**^{201}

Note. First 14 residues were compared with Con A and Con Br. Positions 190–201 of Con A and Con Br from Ref. [17].

Radiolabeled thymidine uptake in growing lymphocytes

The sequence information prompted us to investigate the role of HBSM on lymphoid DNA synthesis. Fig. 4B shows the proliferative activity of HBSM with Con A and PHA as positive control. HBSM at concentration of 2.5 ng/ml induces an increased radiolabelled thymidine

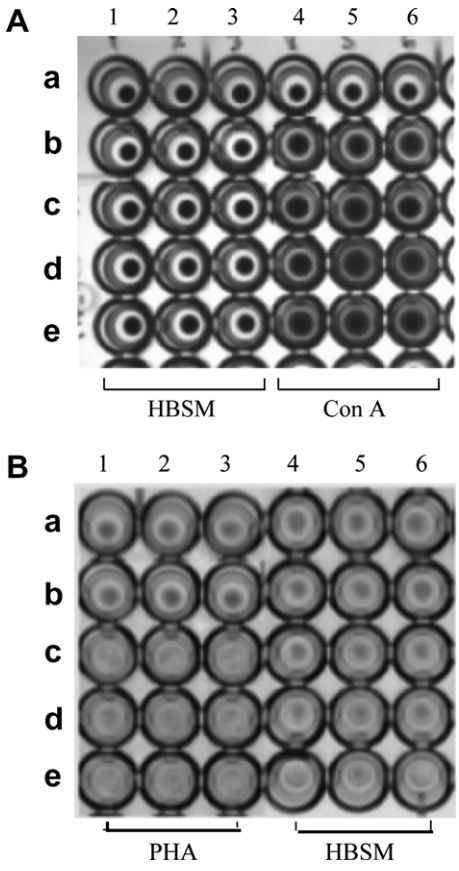

Fig. 3. Cell agglutination assay. (A) Washed SRBC were incubated with different concentration of HBSM or Con A. Wells 1–3 contain HBSM and wells 4–6 contain Con A, with 0.05, 0.1, 0.5, and 1.0 μg in (b), (c), (d), and (e), respectively. Well (a) contains only SRBC. (B) Washed leukocytes were incubated with either HBSM or PHA. Wells (a) contains only leukocytes while wells 1–3, PHA and wells 4–6, HBSM with 0.01, 0.1, 1.0, and 10 μg in (b), (c), (d), and (e), respectively. The cells were incubated at 32 °C for 4 h. Photograph was taken with an Olympus digital camera.

uptake by 83%, whereas Con A and PHA are 85.71% and 65.30%, respectively. The proliferative effect of HBSM was observed to be dose dependent. There was only slight increase in the thymidine uptake at 0.05 ng/ml and a progressive increase was recorded at 0.2 ng/ml of HBSM. The highest [^{3}H]thymidine uptake was noted at 2 ng/ml and no further increase was observed at higher concentration of HBSM (Fig. 4A). The effect of heparin and other sulfated compounds on HBSM proliferative activity was also examined. Fig. 4C shows the proliferative activity of HBSM inhibited by heparin at a ratio of 2:1 and 1:1. Higher ratio of heparin did not show any significant change. Chondroitin sulfate (CS), one of the sulfated GAGs also showed 80% inhibition of HBSM induced thymidine uptake at ratio 1:1. The mitogenic activity of HBSM was also studied after conjugation with G-I and G-ID. These two compounds were purified in-house from the algae *Grateulopia indica*. G-I contains 28% sulfate, while G-ID is the desulfated form. Fig. 4C shows the conjugation of G-I with HBSM inhibited the mitogenic activity of HBSM by 68%. On the other hand, G-ID did not show any inhibition.

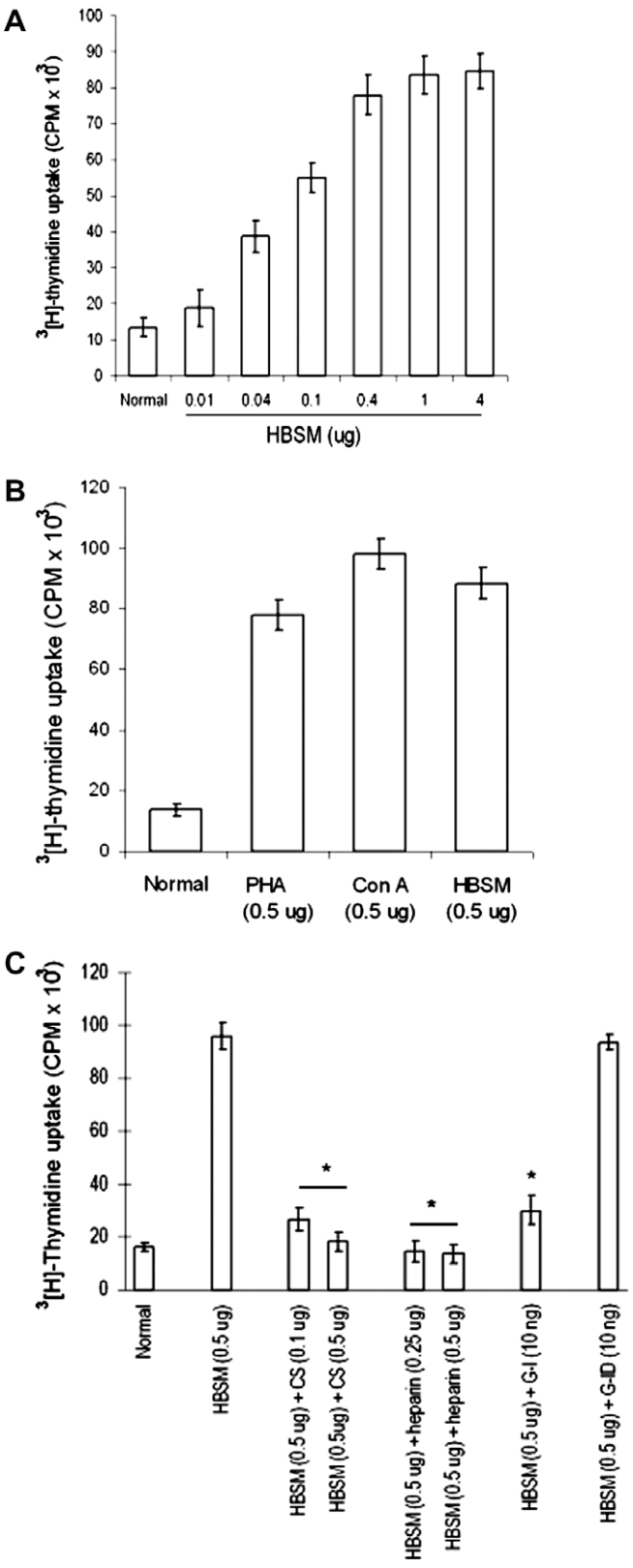

Fig. 4. [^{3}H]Thymidine uptake in growing lymphocytes. Dose-dependent activity of HBSM was highest at 0.4 μg/well (A). Proliferative activity of HBSM with known mitogen; Con A and PHA as positive control (B). Proliferative effect of HBSM inhibited by sulfated compound-Heparin, chondroitin sulfate, and G-I (C). Final volume of each well contains 200 μl ± represents the SD from triplicate well. $^*P < 0.001$.

This result reveals that HBSM induces lymphocytes proliferation in a dose dependent manner with magnitude comparable to known mitogen. However, the heparin-binding sites should remain free in order for HBSM to exert its mitogenic activity.

Discussion

In this paper, we described the amino terminal sequence analysis and biological activities of the heparin-binding sperm membrane mitogen (HBSM). We utilized the major HPLC elute for the N-terminal analysis. The sequence of 14 amino acids revealed by the sequencer was blasted for homology search which revealed 100% identity with the plant lectin; Con A. This lectin was first isolated from *Canavalia ensiformis* (Jack bean) seed and is the best characterized legume lectin and well known for hemagglutination and mitogenic activity [17,18]. Isolation of a heparin binding lectin from human foetal brain that agglutinates erythrocyte has also been reported [19].

Lectin and glycoprotein of special interest are present on the surface of spermatozoa that have role on the spermatozoal maturation and enable fusing with the egg [20–23]. It has also been reported by other laboratories that most growth factors were effectively purified using heparin affinity chromatography [24]. We have earlier reported isolation and purification of HBSM by heparin affinity chromatography and showed that co-incubation of motile sperm cells with antisera in presence of complement initiated a time dependent immobilization of the sperm cell [15]. All these evidence and together with the amino terminal sequence information have led us to investigate the biological activities of HBSM. We assessed both Con A and HBSM mediated erythrocyte agglutination and found that the lectin, Con A (0.5 ng/ml) was effectively agglutinate erythrocyte while there was no sign of agglutination by HBSM. Other cells like leukocytes and yeast (*S. cerevisiae* and *C. albican*) were also not agglutinated by HBSM. Therefore, it is unlikely that HBSM involved in cell agglutination. However, it is not clear whether HBSM can agglutinate other types of cells like the myoloma cell. It could be possible that HBSM contain no complementary saccharides that is responsible for SRBC agglutination and this is a possible explanation for the functional difference between Con A and HBSM.

Among other properties, we draw our interest towards the aspect of mitogenic activities and found similar comparable magnitude with Con A and PHA. We assessed the mitogenic property by utilizing the radiolabelled thymidine incorporation on the proliferative lymphocytes. The proliferative effect by HBSM was dose dependent with maximum incorporation obtained at 2.5 ng/ml. Previously, we have observed that HBSM posses strong affinity to heparin, therefore, studies were also directed towards understanding whether there was any effect of sulfated glycans on the mitogenic activity. We used heparin, chondroitin sulfate and an in-house isolated sulfated glycans (known as G-I) in the proliferative assay. Interestingly, we found that heparin conjugated HBSM inhibited the proliferative activity. Similar type of inhibition was also found with chondroitin sulfate and the in-house isolate, G-I. Therefore, it appears that in order for HBSM to exert its mitogenic activity, the heparin-binding site should remain free. It should be noted that in contrast to sulfated glycans, the desulfated form (G-ID) did not inhibit proliferative activity of HBSM. Altogether, our data suggest that the cell proliferative pathway may be different for HBSM and Con A as the latter is not known to interact with heparin/heparin sulfate which are major cell surface receptor for various growth factor/heparin-binding growth factor family; viz; fibroblast growth factor. It has also been reported that, loss of the heparin-degrading endosulfatase (HSulf-1) up-regulates the heparin-binding growth factor signaling in cancer [25]. Interestingly, we also observed by enzyme-linked immuno assay (ELISA), that anti-FGFa recognized HBSM (data not shown).

In conclusion, the result described in this study, showed that in spite of amino terminal sequence identity between HBSM and Con A, there are differences in the biological properties. Unlike Con A, HBSM showed no erythrocyte agglutinating activity. On the other hand HBSM is capable of inducing lymphocyte proliferation with comparable effect. At present, the complete sequence information of HBSM is lacking and works are directed towards the structural analysis.

Acknowledgments

The authors thank Dr. A.K. Sen Sr. for providing G-I and G-ID, the technical assistance rendered by Sudipto Das, and the research fellowship provided to Visesato Mor by University Grants Commission, New Delhi, India.

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