

**EXPRESSION AND CHARACTERIZATION OF RECOMBINANT
BUFFALO BSP PROTEIN AND ITS EFFECT
ON *IN VITRO* FERTILIZATION**



**THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF**

**DOCTOR OF PHILOSOPHY
IN
ANIMAL BIOTECHNOLOGY
BY**

Dr. SUDAM BAG
M.V.Sc. (Animal Biotechnology)

**ANIMAL BIOTECHNOLOGY CENTRE
ICAR - NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA**

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Approved By


Dr. Praveen Malik
CO ADVISOR

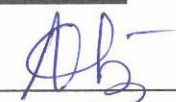
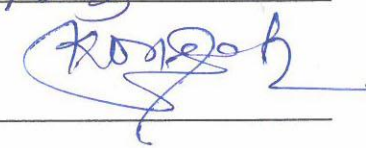
DR. R. K. Sharma


Malakar
16.10.20
Dr. Dhruba Malakar
MAJOR ADVISOR

(
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2. **Dr. Sudershan Kumar**
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(Director's Nominee)



16/10/2020



ANIMAL BIOTECHNOLOGY CENTRE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132 001 (HARYANA), INDIA



Dr. Dhruva Malakar, Ph.D
Phone:01842259541(O)9416741839(M)
Principal Scientist, ABTC, NDRI

E-mail: dhrubamalakar@gmail.com

Date:

CERTIFICATE-I

This is certified that the work embodied in the Thesis entitled **“Expression and Characterization of recombinant Buffalo BSP proteins and its effect on *In Vitro Fertilization*”** is a bonafied research work carried out by **Dr Sudam Bag** for the award of degree of **Doctor of Philosophy in Biotechnology** under my supervision and no part of the thesis has been submitted for any other degree or diploma.

Date:

Dr. Dhruva Malakar
(Research Supervisor)

डॉ. प्रवीण मलिक

Dr. Praveen Malik

पशुपालन आयुक्त

Animal Husbandry Commissioner

Tel.: 23384146



सत्यमेव जयते

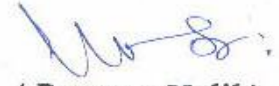
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कृषि भवन, नई दिल्ली-110001

Government of India
Ministry of Fisheries, Animal Husbandry & Dairying
Department of Animal Husbandry and Dairying
Krishi Bhawan, New Delhi-110001

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(**Praveen Malik**)

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Above all, I humbly pay my gratitude to the Almighty who always graced me with his blessings.

Place: Karnal
Date:

Sudam Bag
Dr Sudam Bag
(Registration No- 13-P-BT-13)

Dedicated
To My
Beloved Family
&
Respected Guides

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LIST OF ABBREVIATIONS

%	:	Percentage
°C	:	Degree centigrade / Celsius
µg/L	:	Microgram per litre
µg/ml	:	Microgram per millilitre
µl	:	Microlitre
µmol	:	Micromole
AGE	:	Agarose gel electrophoresis
Amp.	:	Ampicillin
BCIP	:	5-Bromo-4-chloro-3-indolyl phosphate
BLAST\	:	Basic local alignment search tool
BP	:	Base pair
Conc.	:	Concentration
Da	:	Daltons
DEAE	:	Diethylaminoethyl
DNA	:	Deoxyribonucleic acid
DW	:	Distilled water
EDTA	:	Ethylene diamine tetra-acetic acid
G	:	Relative centrifugal force
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
K	:	Kilo
LB	:	Luria-Bertani
M	:	Molar
NFW	:	Nuclease free water
NTA	:	Nitrilotriacetic acid
PBS	:	Phosphate buffer saline
PDB	:	Protein in Data bank
RE	:	Restriction enzyme
WCL	:	Whole cell lysate
X gal	:	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranose

ABSTRACT

Binder of Sperm Proteins (BSPs) the important proteins of seminal plasma. These are involved diverse functions such as capacitation, fertilization and protective action against stressful conditions. In general, all isoforms of BSP protein are having two fibronectins type II (FN2) domains separated by polypeptide linker. Each FN2 domain of BSP is conserved among the BSP family which binds to different molecule like heparin, gelatin, high-density lipoprotein, choline phosphatidyl choline, and glycosaminoglycan. The lower level of the protein in the native form makes the study of its function difficult. This study is aimed to produce the recombinant BSP1 (re-BSP1) in *E. coli* and its purification for understanding the molecular and functional characteristics. The BSP1 gene from buffalo was amplified PCR at 52°C for 30sec using designed primer sets. The 468 bp amplicon was cloned in pEasy cloning vector and transformed into *E. coli* (DH5 α). The recombinant clones were first screened by visualizing its blue-white colour from Luria broth /ampicillin/ X-gal/ Isopropyl β - d-1-thiogalactopyranoside plates then confirmed by colony polymerase chain reaction, restriction enzyme digestion and sequencing. Multiple sequence alignment at nucleotide and protein level using ClustalW2 program revealed different isoforms of BSP of different species and MegavX software was used to reconstruct the phylogenetic tree by maximum likelihood (ML) method and Jones-Taylor- Thornton model with 1000 bootstrap replicates. The positive clones were further subcloned in pET28a vector for transformation in *E. coli* BL21 (DE3) and optimized the condition such as temperature of 16°C and 1 mM IPTG concentration for production of ~17kDa size re-BSP1. In summary, we optimized the protocol for expression of re-BSP1 and successfully produced re- BSP1 in soluble form. The effect of purified re-BSP1 on sperm motility and morphology status of spermatozoa were also observed by CASA study and staining (eosin-nigrosin & trypan blue etc,) and buffalo blastocyst production. It was found that the rec-BSP1 enhanced the sperm motility at a concentration of 50 μ g/ml for 1 h of incubation without having any detrimental effect on the sperm morphology and a significant ($P<0001$) increase in blastocyst production at concentration of 50 μ g/ml rec-BSP1. Hence this finding represents a new insight and advance the prospective approach to develop a potential fertility factor in reproduction.

सारांश

मुख्यतः मादा पशु जानवर की प्रजनन सफलता नर पशु की प्रजनन क्षमता पर निर्भर करती है। कृत्रिम गर्भाधान (AI) और कायोप्रेजर्वेशन पशुधन में प्रजनन क्षमता को बढ़ाने के लिए एक नर पशु की अनुवंशिक क्षमता का उपयोग करने के लिए बहुत महत्वपूर्ण है। वीर्यपात वीर्य के प्लाज्मा में कई प्रोटीन होते हैं जो एक बैल में प्रजनन क्षमता के स्तर को प्रभावित करते हैं। Mc cauley et al. (1999) उपकल्पित किया कि उप-उपजाऊ शुक्राणु की उर्वरता सक सांड के मलबे से लिए गए सेमीनाल प्लाज्मा के साथ इलाज करके सुधार कर सकते हैं। शुक्राणु प्रोटीन (बीएसपी) की बाइंडर सेमिनल प्लाज्मा के प्रमुख प्रोटीन में से एक है जो एक बरमा के प्लाज्मा में है लगभग 70 प्रतिशत टोटल सेमीनाल प्लाज्मा प्रोटीन का योगदान देता है। बीएसपी में बहुमूत्रीय आइसोफॉर्म में मौजूद और एक अद्वितीय संरचना है और तनावपूर्ण स्थितियों के खिलाफ क्षमता, निषेचन और सुरक्षात्मक कार्रवाई जैसे विविध कार्य हैं। ऐसा माना जाता है कि यह उच्च शिथिलता वाले शुक्राणु के चयन में भूमिका निभाता है, जो सफल निषेचन की ओर ले जाता है। भयावह भैंस पुनः बीएसपी संयोजक विटो निषेचन (IVF) के उपयोग पर कोई रिपोर्ट उपलब्ध नहीं है। देशी प्रोटीन की प्रजनन क्षमता कम होने की दर को ध्यान में रखते हुए वर्तमान जांच पुनः संयोजन बीएसपी प्रोटीन का उत्पादन करने के लिए प्रस्तावित किया गया था और इसके शानदार कैपासिटेशन (Capacitation) संयोजक विटो निषेचन (IVF) में दिखाया गया था।

प्राइमर फुल लेंथ जीन (*Bubalus bubalis*) BSP mRNA पूर्ण CDS (Accession no. KR703587) के लिए तैयार किया गया था कुल RNA को सेमिनल पुटिकाओं से निकाला गया था और RNA को यादृच्छिक हेक्सामर पाइमर का उपयोग करके संश्लेषित किया गया था। लक्ष्य अनुक्रम के पीसीआर (PCR) प्रवर्धन सीडीएनए का उपयोग टेम्पलेट (Template) के रूप से किया गया था। और सेट प्राइमर के द्वारा PCR किया गया था। 468bp के आकार वाले एम्प्लिकॉन (AMPLICON) को जेज से शुद्ध किया गया था और pEasy blunt vector में चिपकाया गया था सकारात्मक recombinant क्लोनों को प्लेट्स और (LB-ampicillin/X-gal/IPTG) प्लेट्स और colonies से कलर द्वारा स्क्रीन पर (blue white screening) रखा गया था। पीसीआर और आरई पाचन (RE digestion, sequencing) द्वारा पुष्टि का एक अनुक्रम है। BSP जीन के न्यूक्लियोटाइड अनुक्रम विश्लेषण से पता चला कि यह BSP sequence 99 % (with Identity) पहचान के समान था। कम विश्लेषण से पता चला कि प्रोटीन का एन-टर्मिनल अत्यधिक सुरक्षित है जबकि सी-टर्मिनल अलग था। चयनित सकारात्मक क्लोन बाद में अभिव्यक्ति वेक्टर (pET28a) क्लोन किए गए और सी में बदल गए। ई-कोलाई (*E.coli*) सेल और पुनः संयोजन प्रोटीन के उत्पादन के लिए 1mM आईपीटीजी के साथ 16°C पर 20–22 घंटे तक ऊष्मायन अवधि पर रखा गया एवं यह देखा गया कि पुनः संयोजक प्रोटीन का मानकृत अधिकतम उत्पादन इन्ही परिस्थितियों में हुआ था। पुनः संयोजक प्रोटीन 6 हिसटैग प्रोटीन के एन-टर्मिनल पर चला गया इसलिये पुनः संयोजक प्रोटीन निन्टा प्रणाली (NINTA system) द्वारा शुद्ध किया गया था। अगारोज (PAGE)

स्तम्भ के द्वारा एक घुलनशील और सक्रिय प्रोटीन Thioredoxin के रूप में प्रोटीन BL21 (DE3)p_{lysS} कोशिकाओं में उत्पादन किया गया था । प्रोटीन के 16.2 KD आकार की पुष्टि साथ एसडीएस पृष्ठ एवं Western blotting के द्वारा जाँच की गयी। पुनः संयोजक प्रोटीन घुलनशील रूप में अधिकतम 2 मिलीग्राम प्रोटीन के रूप में प्राप्त किया गया था। पुनः सजॉयक बीएसपी के इन विट्रो कार्यात्मक अध्ययन भैंस भ्रूण के उत्पादन के लिये प्रोटीन बाहर किया गया और इन विट्रो स्थिति में भैंस भ्रूण के उत्पादन में काफी वृद्धि हुई। पुनः संयोजक बीएसपी isoform को सफलतापूर्वक अलग किया गया और एक प्रोकैरियोटिक (Prokaryotic) अभिव्यक्ति में व्यक्त किया गया। इस प्रणाली और पुनः संयोजक प्रोटीन ने भैंस भ्रूण के उत्पादन में काफी वृद्धि की । और इन विट्रो कार्यात्मक अध्ययन में भैंस भ्रूण के उत्पादन के लिए प्रोटीन बाहर किया गया और इन विट्रो स्थिति में भैंस भ्रूण के उत्पादन में काफी वृद्धि हुई। निष्कर्ष में अपेक्षात्मक 14 प्रमुख पुनः संयोजन सफलतापूर्वक अलग किया गया और एक प्रोकैरियोटिक अभिव्यक्ति में व्यक्त किया गया प्रणाली और इस पुनः संयोजक प्रोटीन ने भैंस भ्रूण के उत्पादन में काफी वृद्धि की । केव शब्द। ऐ एसडीएस पेज पश्चिमी।

CHAPTER –1

Introduction

INTRODUCTION

Reproductive efficiency of an animal population is generally concern about female parent that mostly affects productivity and profitability of commercial cattle and buffalo dairying. In India buffalo population of around 56%, (Economic survey, 2002-2003), which contributes more than 55% of the total milk production. Many more progress is done on cow to increase its fertility but it is still lacking to increase bull reproductive efficiency. De Jarnette reported that reproductive failure is not due to problem in cow rather problem in semen quality. The reproductive success of female animal depends upon fertility of sire in which they inseminate, so assessment of fertility status of sire is also equally important. AI and cryopreservation, this tool is very important for using the sire genetic potential to enhance fertility level of male animal but not up to mark. Ejaculated sperm possess non-cellular portion (Seminal plasma) which consist of factors that influence the level of fertility in bull. The seminal plasma proteins have positive effect on fertility (Bellin et al.,1998; Henault and Killian.,1996). Mc Cauley and co hypothesized that fertility of sub fertile spermatozoa could improve by treating with seminal plasma taken from a fertile sample. Seminal plasma consists number of factors among them BSP (binder of sperm protein) contribute 60-70% of total seminal plasma proteins. BSPs have a unique structure which consists of two fibronectins type II (FN2) domains separated by short chain peptide linker that is conserved among BSP family (Hedrick et al., 2007; Manjunath et al., 2009). Each FN2 domain of BSP is conserved among the family and characterized by binding to different molecule like heparin, gelatin, HDL, choline PL, glycosaminoglycan (Gwathmey et al.,2006). The binder of sperm proteins has more binding affinity to phosphoryl choline moiety of spermatozoa membrane specifically bind to acrosome & mid piece region. The binding is induced confirmation change in the protein which in turn induce cholesterol efflux, capacitate sperm & trigger acrosome (Therienet et al.,1999). The BSP function as molecular chaperone on *in-vitro* condition that help in protein folding in sperm capacitation pathway (Sankhala and Swamy,2010). Manjunath and Therien., (2002) reported that epididymal sperm exposed to BSP proteins and heparin or HDL showed higher acrosome reaction level in BSP protein compared to hepain or HDL.

Seminal plasma (SP) has important role in spermatozoa metabolism (Caballero et al.,2012; Muino-Blanco et al.,2008) in terms of viability (Ashworth et al.,1994; Maxwell

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et al., 1996), motility (Bass et al.,1983; Bernardini et al.,2011; Graham,1994) and cryo-protective in different seasons (Berger et al.,1985; Leahy et al.,2009). Ram SP proteins has ability to prevent cryo-injury w.r.t. seasonal difference (Perez-Pe et al.,2001a) and also protect sperm against stressful events (Cardozo et al.,2006; Marti et al.,2007).

BSP proteins can stimulate cholesterol efflux and phospholipid efflux with dose and time dependent manner (Manjunath.et al.,2002). Again, with concentration of $>20\mu\text{g/ml}$ for cholesterol efflux and $>40\mu\text{g/ml}$ for PL efflux of BSP proteins is more significant. Miller & coworker reported that heparin binding proteins has positive effect on *in-vitro* capacitation.

BSP proteins binding to epithelium of lower oviduct where it forms a sperm reservoir which meant for preserving viability, motility and fertility of sperm (Pollard et al.,1991, Gwathmey et al.,2006, Suarez 2007). The reservoir serves to reduce incidence of polyspermy fertilization because of slow release of sperm during preovulatory period where as oocyte have many more mechanism for blocking polyspermy (Hedrick.2007; Wortzman-Show et al.,2005). The reservoir is also believed that it plays a significant role in selection of high quality sperm that leads to successful fertilization. However, no experimental evidence is available on the use of buffalo BSP in *in-vitro* fertilization.

The native purification of PDC-109 protein from buffalo was done by conventional method which is antigenically similar to BSP protein in buffalo (Manjunath et al., 1987; Hiron et al., 2009).

The availability of pure BSPs can help in the studies related to improvement of fertilization and cryopreservation of spermatozoa of buffalo for faster multiplication of superior germplasm. The paucity of tissue sample is a main hurdle in the purification of BSPs in its native form. The recombinant expression of BSPs is an appropriate alternative to the native pure proteins provided the recombinant proteins are properly folded. There are no available reports on the use of buffalo rec-BSP1 in *in vitro* capacitation and embryo production.

Therefore, the present study was envisaged to understand the mode of binding of buffalo rec-BSP1 on heparin and PC which play a significant role in capacitation of spermatozoa and effect on *in vitro* embryo production (Pollard et al.,1991). The several computational approaches such as molecular modelling, docking, MD simulation, and binding free energy estimation were taken into consideration to understand the structural

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integrity of rec-BSP1 and mode of binding with these ligands. Keeping in view of lower rate of fertility in buffaloes, the present investigation has been conducted to develop a method for production of recombinant BSP in *E. coli* in soluble form and establishing its role in modulation of sperm morphology and function and evaluated its effect on *in vitro* buffalo embryo production.

Moreover, keeping in view the lower rate of fertility as compared to bovine, the present investigation is proposed to conduct molecular cloning and expression and purification of recombinant buffalo BSP and evaluate its effect on *in-vitro* fertilization study. With reference to above points the present research has been done following objectives:

- 1) Molecular cloning and identification of various isoforms of buffalo BSP
- 2) Recombinant expression and purification of relatively predominant buffalo BSP isoform in heterologous expression system.
- 3) Effect of recombinant buffalo BSP on *in-vitro* capacitation and *in-vitro* fertilization

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

A brief resume of work done in India and Abroad

Hiron et al., (2009) tried to identify and isolated the PDC-109 protein in buffalo by conventional method but failed. They produced the antibody against purified PDC-109 protein and identified a novel protein which is antigenically similar to BSP protein in buffalo.

Singh M. et al., (2014) reported the effect of PDC-109 concentration on post thaw cholesterol content and semen quality parameter (SQP). They observed that total seminal plasma protein is similar in freezable and non-freezable condition, however HBP content is more in case of non-freezable whereas cholesterol content is higher in freezable one. They concluded that due to presence of higher concentration of the pdc-109 protein stimulate more cholesterol efflux during cryopreservation.

Swamy et al., (2004) had done different studies x-ray diffraction, ESR and SPR to observed different type of interaction between BSP proteins with spermatozoa and they demonstrated the role of PDC-109 in cholesterol efflux from spermatozoa plasma membrane concomitant to capacitation and subsequently fertilization.

Anabazhagan et al., (2011) demonstrated the interaction of PDC-109 in PL membrane by isothermal titration calorimetric studies and they observed that the binding process is due to hydrophobic interaction and water structure plays a vital role and the affinity increase with temperature upto certain limit.

Anabazhagan et al., (2008) investigated the interaction of PDC-109 in PL by fluorescence approach and quenching of ligand binding shield Trp residue and protein bind to PC containing membrane. They suggested that Trp 90 of PDC-109 is due to hydrophobic interior of choline PL membrane.

Arangasamy et al., (2005) isolated the HBP & GBP proteins using affinity column chromatography and characterized by *in-vitro* fertility test (BCMPT & HOST) and they observed that addition of 40µg/ml of seminal plasma protein improved *in-vitro* function of cauda spermatozoa of buffalo.

Ramteke et al., (2014) isolated HBP and it was purified from heparin agarose affinity column and concentration was estimated by Lowery method and electrophoresis to check protein profile of two bulls. They concluded that TSPP and HBP variation in concentration w.r.t initial sperm quality and presence of HBP (31KDa) more in high fertility bull.

Historical background of BSP

BSP proteins previously thought to be inhibin for its both stimulatory and inhibitory actions on gonadotropins secretion (Manjunath, 1984). BSPA1/-A2 previously called as PDC109 as per nomenclature system of Tatemoto and Mutt (1980). PDC109 was named according to their N and C terminal having aspartic acid and cysteine with 109 amino acids. Manjunath and Sairam (1987) reported BSPA1/A2 (PDC-109), BSPA3 and BSP30, these protein secreted from seminal vesicle. Some factors in seminal plasma which are either may be proteins or non- proteins that influences metabolism, motility (Peitz, 1988) and fertility of spermatozoa (Kulkarni et al., 1996).

Biochemical structure of BSP

The structure of BSPs consist of N-terminal O-glycosylated polypeptide extensions of variable length (23-28 aa) followed by two fibronectin (¹Fn-II, ²Fn-II) (≈40 aa) homologous domain similar to fibronectin domain of collagen (Calvete et al.1996b) conserved with in BSP family (Murrey and Smith, 1997). 1Fn2 and 2Fn2 domains are separated by a short polypeptide linker consist of seven amino acid residues. Each domain consists of four cysteine residues, which forms two disulfide bridges between cysteine to cysteine residues. Hence, each BSP protein contains four disulfide bonds. The structure characterized by binding to the heparin (Cardin and Weintraub,1989), gelatin (Manjunath et al., 1987), high density lipoprotein (HDL) (Manjunath et al., 1989), choline phospholipids (Desnoyers and Manjunath, 1992), and LDF of hen's egg yolk (Therien et al.,1997) glycosaminoglycans (Therien et al., 2005).

The molecular weight of BSP-A1, BSP-A2 and BSP-A3 are 12 -15 kDa and BSP30 protein is 30 KDa (Manjunath et al., 1987). BSP-A1 protein contains one whereas BSP-30-kDa six trisaccharide but BSPA2 contains one disaccharide and all carbohydrate moieties are linked through hydroxyl groups of threonine (O-glycosidic linkage). The PDC-109 protein with size of 14–15 kDa in buffalo (Arangasamy et al., 2005) and cattle (Srivastava, 2011) was observed on SDS-PAGE. Desnoyers, I., and

Manjunath, P. (1993) reported the cation- interaction between the quaternary ammonium group and the aromatic indole ring of a conserved core tryptophan residue which means for binding specificity to choline.

Bovine seminal plasma protein (BSP) family groups are normally glycoproteins with the exception of BSPA3. BSP-A1 composed of neutral sugars, galactosamine, and sialic acid, but neutral sugar absents in BSP-A2 (Manjunath et al, 1987) and both have identical amino acid sequence, with their difference in the degree of glycosylation (Esch et al, 1983). BSP-30-kDa is the most glycosylated member of BSP family. These gelatin-binding proteins in case bull secreted from the seminal vesicles (Manjunath et al, 1987). Salosis et al, (1999) reported that BSP protein mRNA expression in seminal vesicle and ampulla.

Bovine seminal plasma (BSP) proteins contribute around 40-57% of total seminal plasma protein (25- 47% of BSP-A1/-A2, 3-5% of BSP-A3, and 3- 7% of BSP-30 kDa) and 4- 6% of sperm total protein (2.5- 4% of BSP-A1/-A2, 0.4-0.9% of BSP-A3, and 0.5- 1% of BSP-30-kDa. BSP proteins with concentration of 31.4-46.7 mg/ml present in the seminal plasma of pure bred Holstein bulls (Nauc and Manjunath, 2000).

The percentage of identity between the fn2 domains of the three BSPs w.r.t. their amino acid sequence is 71.4% between PDC-109 and BSP-A3, 53.8% between PDC-109 and BSP-30k-Da, where as 57.5% between BSP-A3 and BSP-30-kDa respectively.

Different functional role of BSP

BSP proteins not only decapacitate the spermatozoa but may eventually promote capacitation (Manjunath et al, 1993). Lane et al (1999) showed that heparin and HDL induce capacitation by different mechanisms. Therien et al. (1997) reported that purified human serum HDL induces capacitation of bovine ejaculated sperm more than that of epididymal sperm. So, some factors present in seminal plasma that accelerate the sperm capacitation process triggered by HDL. Bovine seminal plasma (BSP) one of these factors among them which will be implicated in metabolism of spermatozoa (Manjunath et al, 1993).

The PDC-109 and other two BSPs, plays vital role in sperm storage in the female (Gwathmey et al, 2006). Once capacitated, epithelium therefore the During capacitation, binding affinity of sperm lose for the oviduct and release of sperm from the reservoir. (Gwathmey et al, 2006). PDC-109 also functions as a molecular chaperone *in-vitro*

mainly for folding of proteins which is important in sperm capacitation pathway (Sankhala and Swamy, 2010).

Heparin along with PDC-109 induced sperm capacitation (Sankhala et al., 2011). Manjunath et al, (2002) also suggested that more the concentration of bovine seminal plasma (BSP) proteins in seminal plasma, more efflux of cholesterol and phospholipids result in destabilization that decreases sperm resistance to cold shock and freezing. HDL also stimulated cholesterol efflux in a dose-dependent manner from sperm and the combined effect of BSP proteins and HDL was higher (Thérien et al., 1999). It was also seen that exposure of BSP proteins along with heparin or HDL trigger higher incidence of acrosome reaction as compared with heparin or HDL alone.

Seminal plasma proteins have several functions, including capacitation and acrosome reaction (Thérien *et al.*, 1995; Siciliano *et al.*, 2008). Accessory sex gland fluid (Moura *et al.*, 2006a) and cauda epididymal fluid (Moura *et al.*, 2006b) is related to the fertility of dairy bulls which in turn *in-vitro* penetration of bovine oocytes (Moura *et al.*, 2007a). It has been reported that fertility-associated proteins from bull accessory gland fluid (AGF) specifically, BSP-30kDa and BSP-A1/A2 (Killian *et al.*, 1993; Moura *et al.*, 2006a) are able to bind to membrane of ejaculated sperm. This protein secreted from the vesicular glands (Manjunath *et al.*, 1988) and interacts with the sperm during ejaculation (Desnoyers and Manjunath, 1992), a step that initiates sperm capacitation (Thérien *et al.*, 1995).

Role of BSP proteins on capacitation

Ejaculated semen containing spermatozoa undergo an obligatory maturational process known as capacitation in female genitalia which allows spermatozoa to undergo the zonapellucida induced acrosome reaction (AR) and fertilize oocytes. The heparin increase calcium, pH, and cAMP, seems to be necessary to initiate signaling pathway to induce capacitation (Galantino-Homer et al., 1997) and decrease in the membrane cholesterol: phospholipids ratio (Ehrenwald et al., 1988; Pommer et al., 2003).

PDC-109 initiate capacitation with dose dependent manner in presence of promoters such as HDL and heparin (Therien et al., 1995) and trigger acrosome reaction (Arangasamy et al., 2005). The AR also exposes a second receptor on sperm that binds to zona pellucida which allows the release of hydrolytic enzymes and the penetration to ZP

and this interaction results in gamete fusion (Go KJ, Wolf DP. (1983), Meizel S. (1985), Oliphant G, (1985), Langlais J, Roberts KD. (1985), Yanagimachi R. (1988).

Role of BSP proteins on fertilization

The secretions from accessory sex gland on sperm have both beneficial and harmful effect on fertility of sperm (Chang, 1957; Gaur and Talwar, 1975; Reddy *et al.*, 1979; Corteel, 1980; Sadowski and Rogers, 1985; Shivaji and Bhargava, 1987). It was reported that penetrations of sperm from low fertility bulls were improved by treated accessory gland factor (AGF) of high fertility bull (Henault and Killian, 1996).

Molecular cloning and Expression of BSP protein

Jasmine et al., (2009) successfully, producing recombinant human sperm binding protein(BSPH1) and they used the purified protein in establishing the biological role such as binding ability to phosphoryl choline liposome, sperm capacitation, motility and viability.

Lefebvre et al., (2009) successfully expressed & purified recombinant human sperm binding (BSPH1) soluble, active, his (6) thioredoxin tagged protein in origami B(DE3) pLysS cells which was accumulated in inclusion body while expressed with N terminal hexa histidine tag in BL21 (DE3) E coli cell.

Li-Ping et al., (2012) studied on molecular cloning, purification and functional characterization of a newly isolated protein from swine seminal plasma named as swine PEBP4 (sPEBP4) which is secreted from the epididymis and Functional analysis of this protein demonstrated its ability to stimulate sperm motility.

Plante et al., (2012) amplified cloned the gene murine binder of sperm protein homolog1 and expressed it in E coli which was characterized by Western blot by using anti murine BSPH1 antibody. They also compared the effect of native protein vs recombinant protein in capacitation.

Edith et al. (2013) characterized and expressed the ram seminal vesicle protein (RSVP14) in *Pistia pastoris* and they suggested that the protein might have similar role in capacitation and protective effect against cold shock injury.

Serrano et al., (2013) studied on characterization and expression of ram seminal plasma protein (RSVP14) and its effect to protect spermatozoa against cold shock injury. The protein was obtained from insect cell extracts & in E coli and detected in both pellet

& supernatant. They concluded that the expressed sequence contains two FN2 domain & signature characteristic of bovine seminal plasma protein family and expressed protein against the coded sequence has similar role sperm functionality.

Bustamante-fillo et al., (2014) had done recombinant expression and purification in E coli and they conducted two experiment Western blot and Immunofluorescence microscopy analysis and observed its effect on binding to spermatozoa.

Plante et al., (2014) studied on characterization of human binder of sperm Homolog1 (BSPH1) protein and evaluated its effect on different sperm function such as sperm motility, acrosome reaction. They concluded that they protein share many biochemical and functional characteristics with BSP secreted by seminal vesicles of ungulate and behave similar to its murine epididymal orthologue BSPHI.

Rodríguez-Villamil et al., (2016) had evaluated functional aspects of binder of sperm 1 (BSP1) in the bovine species i.e. they studied the effects of purified BSP1 from both SP of Bos taurus bulls and SVF of Bos indicus bulls on bovine *in-vitro* embryo development after fertilization with ejaculated and epididymal sperm and they concluded that purified BSP1 from bull seminal vesicles was able to bind to and induce capacitation of ejaculated and epididymal sperm and BSP1 added to fertilization media and allowed proper cleavage and embryo development, with the effects being modulated by previous exposure or not of spermatozoa to seminal plasma.

El-Shahat et al., (2018) had studied the influence of different concentrations of oviduct fluid (OF) or follicular fluid (FF) on ram sperm capacitation and acrosome reaction *in-vitro* and concluded that addition of 50 mL/mL of oviduct fluids had increased in individual motility percentage and effect on sperm hyperactivity after 1-2 h incubation and maintained significantly ($P < .05$) higher total acrosome reaction (AR %) after 3 h of incubation.

Patil, S., et al. (2018) studied on localization of PDC-109 by Indirect immunofluorescence one of the fertilities regulating proteins in buffalo spermatozoa. They concluded that PDC-109 localized in the post acrosomal region which indicate that PDC-109 might be involved in the sperm penetration deep into the oocyte cytoplasm.

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

The present study was undertaken to isolate the confirmed sequence genome and cloned into expression vector to produce recombinant BSP1. Then evaluated its effectiveness as a media supplemented with rec-buBSP1 in In Vitro studies for increasing capacitation process and production rate of cleavage/blastocyst on in vitro fertilization study. The glasswares used in the proposed study were made of high grade and were thoroughly cleaned & sterilized one. All laboratory reagents and solutions were prepared in nuclease free water/ de-ionized/ distilled water. Chemicals and molecular biology reagents were of Anal/LR/ Molecular Biology grade purchased from reputed suppliers/manufacturers. Different instruments such as zoom stereomicroscope (Nicon Japan), inverted microscope (Nicon Japan), CO₂ incubator (Thermoforma Scientific, USA), Horizontal Agarose electrophoresis unit (Consort-EV2310, Belgium), Gel dock (Syngene, USA), Thermocycler (Long gene MG96G), Centrifuge (Eppendorf), Nano Drop 1000 (Thermo Scientific), vertical SDS PAGE unit (Bio Rad Lab, India), His Trap HP cartridge (GE health care) etc. were used for the proposed study. Composition of buffers, stock solutions, bacteriological media, and sources of materials, chemicals, reagents, media used are given in *Annexure*.

Methods: Objective 1: Molecular cloning and identification of various isoforms of buffalo BSP

3.1 Total RNA Isolation

Total RNA was extracted directly from the tissue samples (seminal vesicles) by using Trizol reagent (Cat no: # 79306/Qiagen) as per manufacturer's protocol. The mixture was allowed to stand at RT for 5-10 min. The mixture was shaken vigorously for centrifuged at 12000 rpm for 5-10 min at 4⁰C. The mixture separated in two lower red, phenol chloroform phase and a colourless upper aqueous phase. The aqueous phase was transferred into a fresh tube and RNA was precipitated by 0.5 ml of 2-propanol at RT for 10 min. Then it was centrifuged at 12000 rpm for 8 min at 4⁰C. The supernatant was discarded and the RNA pellet was washed once with 1 ml of 75 % ethanol. The sample was mixed by vortexing and centrifuged at 12000 rpm for 5 min at 4⁰C. Thereafter, air dried RNA pellet was re-dissolved in 20-30µl of DEPC-TE and stored at -

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70⁰C until further use. RNA samples were further checked on 1.5% denatured agarose gel. For quantitative and qualitative analysis, RNA samples were checked on Nanodrop spectrophotometer by measuring the absorbance at 260,280 and 230 nm. The schematic representation for total RNA isolation has been shown in Fig.3.1

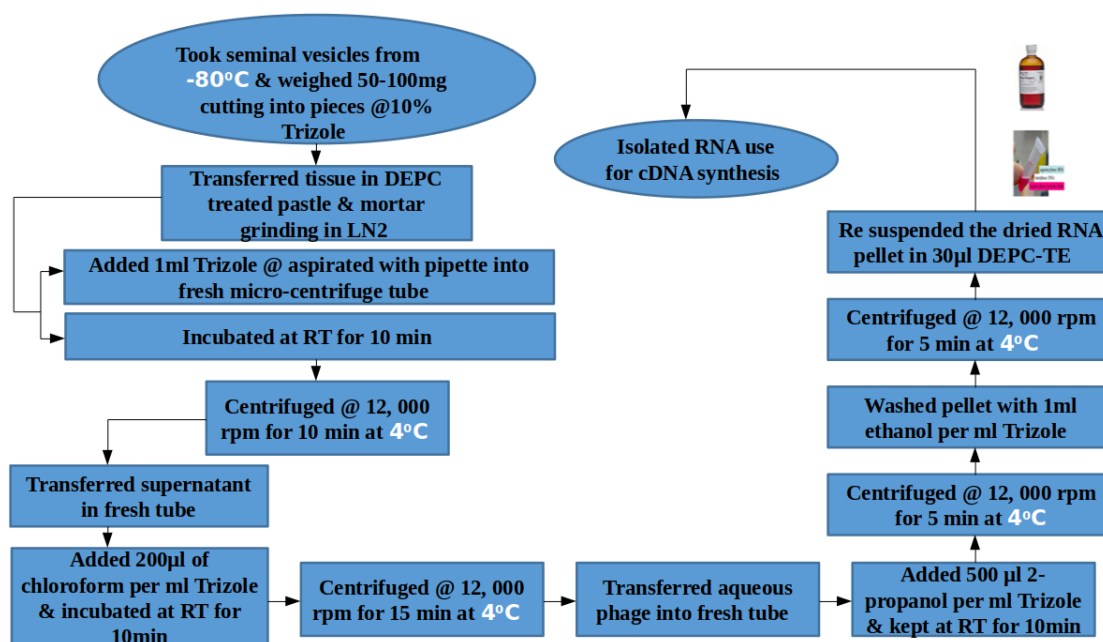


Figure 3.1: Schematic Representation for Total RNA Isolation from seminal vesicles

3.2 Reverse Transcription of seminal vesicular RNA (cDNA synthesis)

Complimentary DNA (cDNA) synthesis was carried out from seminal vesicular RNA using random hexamer primer, as per standard protocol (Cat no: # 205311/Qiagen).

Template RNA (100ng/µl)	5µl
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Random Hexamer (100 pmoles)	1µl
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The mixture was heated to 70⁰C for 5 min and snaps cool on ice for 2 min.

Thereafter, following mixture was added:

5X RT buffer	4.0µl
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RNase inhibitor (20 units µl ⁻¹)	0.5µl
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10mM dNTPs	2.0µl
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RT enzyme (200 units µl ⁻¹)	1.0µl
---	-------

Nuclease free water	2.5µl
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Total reaction volume	10.0µl
-----------------------	--------

The mixture was then incubated at 42⁰C for 1h followed by 95⁰C for 10 min to inactivate the reverse transcriptase.

3.3 Amplification of BSP gene

3.3.1 Polymerase Chain Reaction

The PCR was carried out in standard 25 µl reaction volume using primer pairs, forward 5'- GCGGCG GGATCC CGATGGCACTGCAGTTGGGGCTC -3' and reverse- 5'- GCGGCG CTCGAG GCAATACTTCCAAGCTCTGTCC-3' designed in primer-blast software using *Bubalus bubalis* Binder of sperm 1 (BSP1) mRNA, complete CDS sequence (accession no. **KR703587**) for amplification of BSP gene.

A typical reaction containing the following reagents (Cat no: # 200403/Qiagen) was performed in a 0.2 ml PCR tube:

2X Master mixture	12.5µl
Primer F (10µM)	1.0µl
Primer R (10µM)	1.0µl
cDNA 1.5µl	
Nuclease free water	9.0µl
Total reaction volume	25.0µl

The cycling conditions used for amplifying the BSP were as follows:

Step 1:	Denaturation	94 ⁰ C for 5 min
Step 2:	Denaturation	94 ⁰ C for 30 sec
Step 3:	Annealing	50 ⁰ C -65 ⁰ C for 30 sec
Step 4:	Extension	72 ⁰ C for 30 sec

From step 2 – step 4 was repeated for 35 cycles

Step 5: Final extension 72⁰C for 7 min

3.3.2 Agarose Gel Electrophoresis (AGE) of Amplicons

The BSP amplicons were resolved in a maxi-gel horizontal apparatus using 2 % agarose gel electrophoresis in TAE buffer at 10 V cm⁻¹ gel length for about 1h and visualized by alternative ethidium bromide staining (EZ Vision) in UV transilluminator (Syngene, USA). The approximate size of amplicons was estimated using DNA size marker in one of the lanes. The composition of solutions for agarose gel electrophoresis is given in Annexure.

Briefly, 100 ml of 2 % agarose solution was prepared in TAE buffer by boiling in microwave oven. It was then allowed to cool at 50°C and EZ Vision solution added to a final concentration of 0.5µg ml⁻¹. The molten agarose was poured in the gel tray with a comb inserted in the gel tray towards the cathode end and gel was allowed to solidify at room temperature. Thereafter, electrophoresis tank was filled with TAE buffer and the gel was immersed in buffer and combs were removed. The samples were prepared by mixing 5 µl of PCR products with 1 µl 6x gel loading dye solution (Lot no: # 00539404) and loaded in the wells. The 100 bp plus DNA size marker (Lot no: # 00111110) was also loaded in the well along with the PCR products and electrophoresis was carried out at 100 V for 1h. The gel was visualized in a transilluminator (Syngene, USA).

3.3.3 Purification of PCR products from Agarose Gel

PCR products were purified using Qiagen gel extraction kit following manufacturer's protocol. The PCR products was run in 1.5 % low melting point (LMP) agarose (Lot no: # 1567C077/Life science) gel in TAE buffer along with DNA marker. The gel slice containing the desired DNA fragments were excised using a clean, sharp scalpel or razor blade, on a UV trans-illuminator with minimum UV exposure. The slices were then cut into small pieces and placed into sterile centrifuge tube. Thereafter, 10 µl of membrane binding solution per 10 mg of gel slice was added and incubated at 50-65°C in a dry bath until gel slice completely dissolved. Further, mini elute column was placed on to a 2 ml collection tube provided in kit and dissolved gel mixture was added into the column. The column was then centrifuged at 10000 rpm for 1 min and flow through was discarded. The column was washed with 0.7 ml of membrane wash solution by centrifuging at 10000 rpm for 1 min thereafter, discarded the flow through and repeated the same step with 0.5 ml of membrane wash solution. After washing, the column was centrifuged at full speed for another 1 min to remove residual ethanol

present in column. The column was transferred onto a fresh 1.5 ml centrifuge tube. Finally, the PCR products was eluted in 20 µl of nuclease free water and stored at -20°C until further use.

3.4 Cloning of PCR Products

The purified PCR products were cloned into pEASY blunt cloning vector using TA cloning mechanism (Fig. 3.2). The appropriate amount of PCR products were subsequently cloned into plasmid vector keeping the insert: vector molar ratio to 3:1 as per the formula:

$$\text{ng of insert (PCR product)} = \frac{\text{ng of vector} \times \text{kb size of insert} \times 3}{\text{kb size of vector}}$$

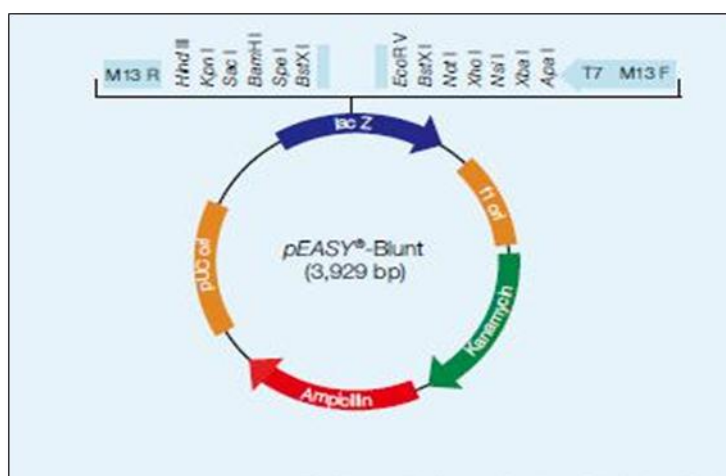


Figure 3.2: Map of pEASY blunt Cloning Vector

3.4.1 Ligation of PCR Products into Plasmid Vector

The PCR products were ligated with desired vector as detailed below: Purified

PCR product	x µl
Vector (55 ng µl ⁻¹)	1.5 µl
10X T ₄ Ligase buffer	1.5 µl
T ₄ Ligase (5 Weiss unit µl ⁻¹)	1.0 µl
Total reaction volume	15.0 µl

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The volume made up to 15 µl by adding nuclease free water. The ligation was performed at 4°C for 20-24 h.

3.4.2 Transformation of Competent DH5α *E. coli* Cells

The ligation mixture (7µl) was added to competent cell mixture and the tubes were flicked gently for homogenous mixing. The tubes were kept for 30 min on ice and then given heat shock by incubating at 42°C for 60-90 sec and immediately transferred to ice for 10-15 min.

Further, 700 µl of Luria-Bertani (LB) (-) ampicillin & kanamycin broth was added to above mixture and tubes were then incubated at 37°C in a shaker water bath at 120 rpm for 30-45 min. Thereafter, tubes were centrifuged at 8000 rpm for five min at RT and supernatant was discarded leaving 150 µl for re-suspending the pellet. The re-suspended transformed DH5α cells were then plated with 150 µl volume/plate on 90 mm LB/ampicillin (100 µg/ml) and kanamycin (50 µg/ml) (+) agar plates containing 40 µl of 100mM solution of IPTG (Isopropyl-thio-Beta-D galactoside) and 40 µl of 20 mg/ml solution of X-gal (5-bromo 4-chloro 3-indolyl-beta-D-galactoside). Finally, plates were incubated overnight at 37°C and thereafter stored at 4°C, before screening of colonies.

3.4.3 Screening and Selection of DH5α Transformant Colonies

The colonies were screened by picking up the colonies from overnight incubated plates. The colonies transformed with recombinant plasmid appear white in colour due to insertional inactivation of *lac Z* gene, as a foreign DNA was ligated in the multiple ligation site. The colonies transformed with re-circularized/linearized pEASY blunt cloning vector appear blue in colour as intact *lac Z* gene lead to the synthesis of galactosidase, which breaks down chromogenic substrate X-gal present in the medium. Further, from each plates, ten randomly selected white and smooth recombinant DH5α colonies were transferred to 500 µl of LB/ampicillin (+) and kanamycin (+) broth into 1.5 ml sterile micro-centrifuge tubes by sterilized loop and kept in shaking incubator at 37°C for overnight. The positive clones were stored at 4°C until use for plasmid isolation.

3.4.4 Isolation of Plasmid

The plasmid DNA was extracted from transformed DH5α cells by the alkaline lysis method (Green and Sambrook, 2012). The composition of solutions required for plasmid isolation is given in Annexure.

In brief, five ml of sterile broth was inoculated with 150 µl of positive clone and kept in a shaking incubator at 37°C for overnight. Thereafter, overnight grown culture was pelleted by centrifuging at 8000 rpm for five min at RT and transferred to centrifuge tube by re-suspending the pellet in 300 µl of ice cold alkaline lysis solution I. Further, 300 µl of alkaline lysis solution II was added and mixed thoroughly by inverting the tube. After that add 300 µl of ice cold alkaline lysis solution III and mixed immediately by inverting the tube 4-6 times. The resultant mixture was centrifuged for ten min at 10,000 rpm and transferred the supernatant into 2 ml autoclaved centrifuge tube. In supernatant equal volume of Phenol: Chloroform (1:1) solution was added and inverted several times. Thereafter, mixture was centrifuged at 10000 rpm for 5 min at RT. Further, aqueous phase was transferred into a fresh tube and plasmid was precipitated by 0.5 ml of 2-propanol at RT for 5 min. The mixture was centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the plasmid pellet was washed with 1 ml of 75 % ethanol by centrifuging at 10000 rpm for 5 min. Thereafter, air dried plasmid pellet was re-dissolved in 30 µl of nuclease free water and stored at -70°C until further use. The schematic representation for plasmid isolation has been shown (Fig. 3.3)

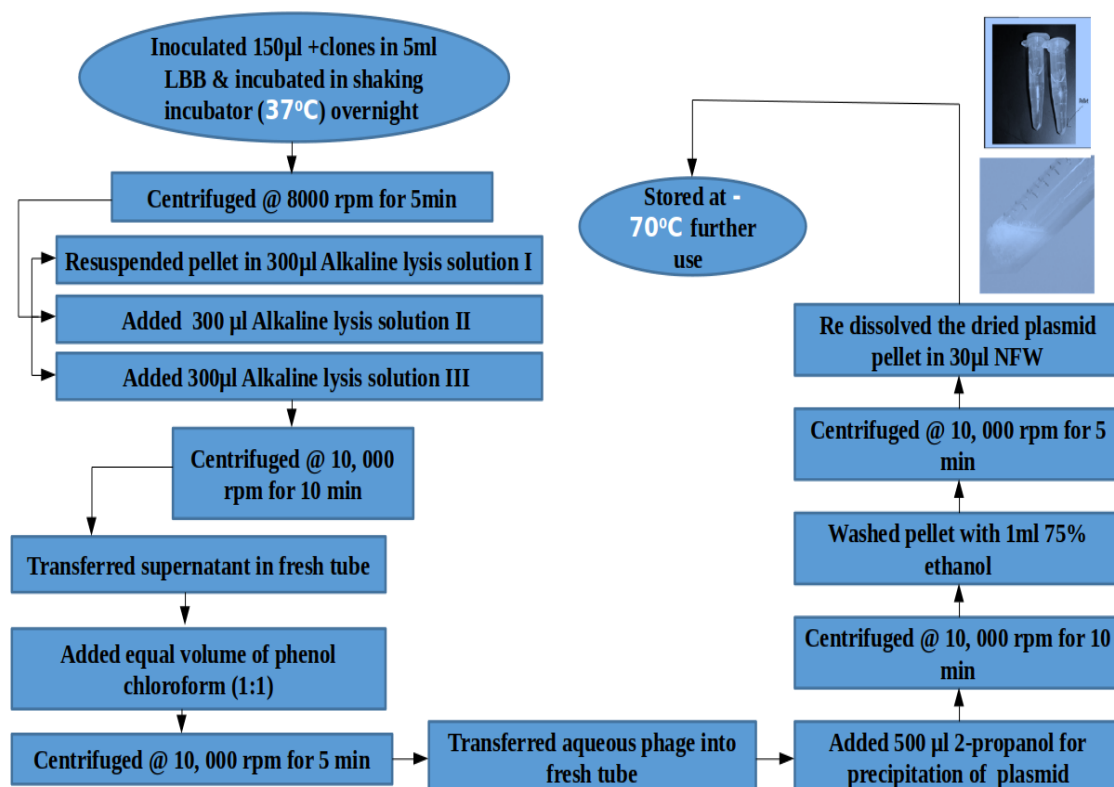


Figure 3.3: Schematic Representation of Plasmid Isolation by Alkaline Lysis Method

3.4.5 Release of Insert from Plasmid

Isolated putative recombinant plasmids was digested with appropriate restriction enzymes and further sequenced to check the presence of desired insert. Recombinant pEASY blunt cloning vector was digested with *Bam*HI and *Xho*I for insert confirmation.

The reaction mixture containing the following reagents was used in RE analysis.

Plasmid DNA	10.0 µl
10X Fast digest green buffer	2.0 µl
BamHI	1.0 µl
XhoI	1.0 µl
Nuclease free water	6.0 µl
Total volume	20.0 µl

The contents were prepared in 0.5 ml centrifuge tube and thoroughly mixed by a brief spin. The tube was then incubated overnight at 37⁰C. The RE digested products were run on

1.5 % agarose gel with DNA marker to check the release of the insert of desired size from recombinant plasmid DNA. The clones, releasing the exact size of insert were selected as positive clones for sequencing purpose.

3.4.6 Sequence Analysis

The sequences were initially analyzed using NCBI online BLAST server to identify the sequence identity. The positives clones were also sent for sequencing by Chromos biotech pvt ltd. DNA sequencing was performed to confirm the identity of insert. Both strands of the insert were got custom sequenced using T7 universal, reverse along with gene specific primer. The nucleotide sequences of the other BSP were retrieved from the NCBI and used for further analysis. These sequences were further used for multiple sequence alignment by ClustalW2 program (www.ebi.ac.uk/Tools/msa/clustalw2) and pairwise sequence identities were determined between the orthologs and paralogs. The construction of the phylogenetic tree was done

by Mega X software (<https://www.megasoftware.net/>). The sequences were aligned by muscle program by using UPGMA method and construction of the phylogeny tree was carried out by using the maximum likelihood method (ML) based on Jones-Taylor-Thornton (JTT) model with bootstrap value of 1000. five numbers of discrete gamma distribution categories were taken into account for evolutionary rates using all sites. The tree was obtained by heuristic search using Nearest-Neighbor-Interchange (NNI) method; however, the initial tree was obtained by Neighbor Joining method in MEGA X program.

Methods: Objective 2: Recombinant expression and purification of relatively predominant buffalo BSP isoform in heterologous expression system

3.5 Sub-cloning of relatively predominant BSP isoform in Prokaryotic expression vector

Based on copy numbers of each transcript obtained from sequence analysis, the relatively predominant BSP isoform was further sub cloned into in prokaryotic expression system for recombinant expression and purification.

3.5.1 PCR amplification of predominant BSP isoform gene

In order to clone and express the BSP was first amplified using primer pairs given below (Table 3.1). Firstly, PCR amplicons were amplified using described primer pairs by PCR in 25 µl reaction mixture as described earlier.

Table 3.1: The Oligonucleotides Pairs Used for PCR Amplification

Primer Codes	Sequences (5'-3')	Region Encoding	Tm (°C)
BSP-1F	5'AGCT CATATG ATGGCACTGCAGTTGGGGCTC3'	400-500 bp	52
BSP-1R	5'CTAGCT CTCGAGG CAATACTTCCAAGCTCTGTCCTT3'		

Forward primers have *NdeI* restriction site while *XhoI* in reverse primers. The restriction sites are shown in bold. The PCR mixture and cyclic condition for amplification of target sequence mentioned below (Table 3.2 & Table 3.3).

Table 3.2: PCR reaction mixture for 50µl reaction,

Components	Volume/concentration
Dream Tag mixture	25µl
Forward primer	0.5µM
Reverse primer	0.5µM
Template DNA	1.0µl
Nuclease free water	Xµl
Total mix	50.0µl

Table 3.3: The Cycling Conditions Used for Amplifying the BSP were as Follows:

Step	Cycle name	Temperature (°C)		Time
1.	Initial denaturation	94		5 min
2.	Denaturation	94		30 sec
		BSP-1	52	30 sec
4.	Elongation	72		30 sec
Step 2 to 4 was repeated for 35 cycles				
5.	Final elongation	72		7min

3.5.2 Agarose Gel Electrophoresis (AGE) of Amplicons

The BSP predominant amplicon was resolved in a maxi-gel horizontal apparatus using 2 % agarose gel electrophoresis as described earlier and visualized under UV transilluminator (Syngene, USA).

3.5.3 Purification of PCR products from Agarose Gel

PCR products were purified using Qiagen gel extraction kit following manufacturer's protocol. The purified product was then quantified by spectrophotometer.

3.5.4 Sub-cloning of BSP's in prokaryotic expression vector

The pET28a for prokaryotic host cell was used for expression analysis of BSP protein (Fig. 3.4).

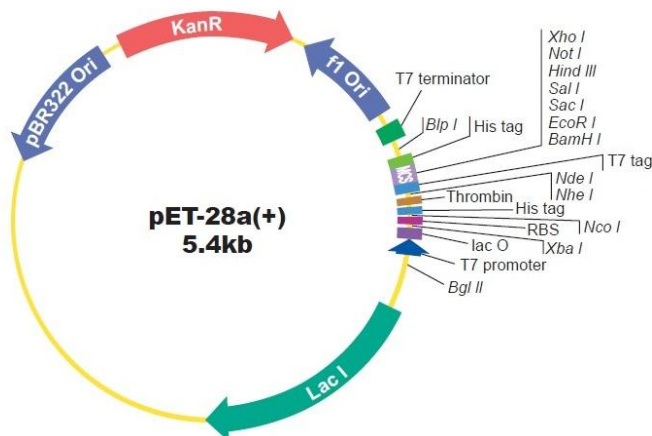


Figure 3.4: Vector Map of Expression Vector pET28a

Both recombinant plasmids pEasy and vector (pET28a) were double digested with restriction enzyme to facilitate directional cloning. The volume of reaction mixture was as follow:

Recombinant plasmid/ vector	10 µl
10X Fast digest green buffer	2 µl
<i>NdeI</i>	1 µl
<i>XhoI</i>	1 µl
NFW	6µl
Total volume	20 µl

The mixture was incubated at 37⁰C for overnight and run on 1 % agarose gel (LMP) in TAE buffer along with DNA marker. Both the digested products (desired insert and vector) were purified by Qiagen gel extraction kit following manufacturer's protocol.

3.5.5 Ligation

The digested insert and vector were ligated using following mixture and the reaction was performed at 4⁰C for 20-24h. The appropriate amounts of inserts were subsequently cloned into expression vector keeping the insert: vector ratio to 3:1

Materials and methods

following the manufacturer's protocol.

<i>NdeI/XhoI</i> released insert for recombinant plasmid	X μ l
<i>NdeI/XhoI</i> digested expression vector	Y μ l
10X T ₄ DNA Ligase buffer	1.5 μ l
T ₄ DNA Ligase	1.0 μ l
Total reaction volume	15.0 μ l

3.5.6 Transformation

Transformation of the construct was carried in competent BL21 (DE3) pLysS strain of *E. coli* and similar fashion as in case of DH5 α for all fragments. The recombinant clones represented as white colonies were picked up and grown overnight by inoculating into 5 ml of LB (+) Kanamycin for PCR selection of positive clones.

3.5.7 Screening of Recombinant Clones

Recombinant colonies were transferred to 500 μ l of LB/Kanamycin (+) broth in sterile centrifuge tubes by sterilized loops and kept in shaking incubator at 37°C for overnight. The cultures were then screened by colony PCR, RE digestion and sequencing for confirming the presence the targeted insert following the optimized PCR protocol.

3.6 Expression of Recombinant Protein (predominant BSP isoform)

3.6.1 Induction of Protein Expression

Induction of protein expression was done according to procedure described by Green and Sambrook (2012). The PCR positive clones was inoculated in 1ml of LB broth (containing 50 μ g/ml kanamycin), where vector transformant of *E. coli* was as a control and incubate the culture at 37°C for overnight. Thereafter, inoculated 10 ml of LB broth (containing 50 μ g/ml kanamycin) with 100 μ l of each overnight culture and incubated for 3 h at 37°C in a shaking incubator until cells reach mid-log growth (A_{550} of 0.5-1.0). Further, 1 ml of each un-induced culture was transferred in 1.5 ml centrifuge tubes and centrifuged at 8000 rpm for 5 min at RT. The supernatant was discarded and store the pellet at 4°C until further use. The remaining cultures were incubated at 37°C to continue the growth and IPTG was added to a final concentration of 1mM for induction. Thereafter, 1ml of induced cultures was collected in 1.5 ml centrifuge tube at each hour

interval up to 6 h and then centrifuged the same at 8000 rpm for 5 min at RT. Further, the pellets were re-suspended in 60 μ l of 1X PBS (pH 7.2) and mixed with equal volume of 2 X loading buffer of SDS-PAGE. Samples were loaded on gel and electrophoresis was carried out at 100 V till the tracking dye reached the separating gel. After completion of electrophoresis, gel was stained with 0.1 % Coomassie brilliant blue (CBB-R 250) and destained. The gels after visualization were kept in 7.5 % acetic acid solution in water. The schematic representation for induction of recombinant clones has been shown (Fig. 3.5).

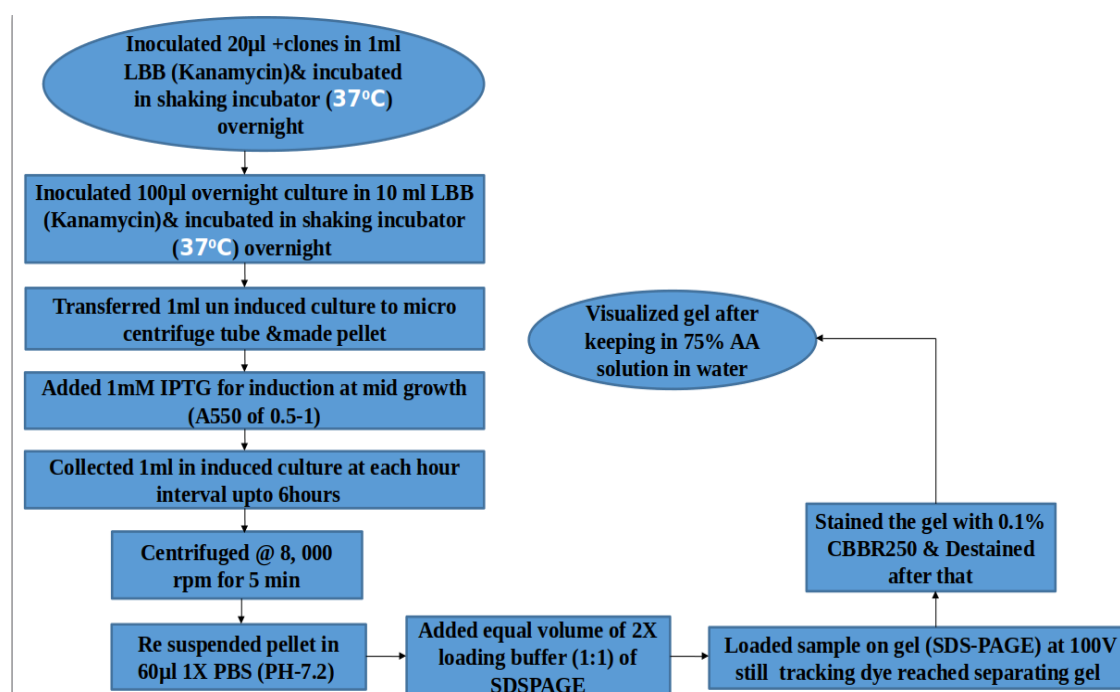


Figure 3.5: Schematic Representation for Recombinant Clone's Induction

3.6.2 Optimization of Protein Expression

Kinetics of expression studies using different concentration of IPTG (1 mM and 2 mM) were carried out to find maximum expression of recombinant proteins. Similarly, attempts were made for expression of rec-buBSP1 in E coli BL21 (DE3) in soluble fraction. Solubility optimization was carried out in 16⁰C temperature for duration of 16-20 hours. The resulting pellet obtained from the culture grown at 12⁰ & 16⁰C were suspended in lysis buffer (@10 μ l lysozyme/ml) and subjected to sonication and run in SDS-PAGE.

3.7 Purification of rec -BSP Proteins by Ni-NTA Agarose/dialysis

The recombinant BSP1 protein was purified using Ni-NTA superflow agarose (Qiagen). Briefly, 50 ml of cell culture pellet was suspending 5 ml of lysis buffer and incubated for 1 h at 37⁰C with occasionally mixing after 10-15 min and subjected sonication. The lysate was centrifuged at 6000 rpm for 30 min at RT and stored the supernatant at 4⁰C till further use. Thereafter, placed a sintered disc at the base of 5 ml syringe and add 2 ml of Ni-NTA agarose from the top. Equilibrated the column with 3 volume of lysis buffer. Supernatant was incubated with Ni- NTA agarose for 1 h at room temperature (RT) in the presence of 20 mM imidazole and packed into the column. Collected the flow through in tube and store at 4⁰C and unbound proteins were washed sequentially with 3 volume of wash buffer in presence of 10, 15, 20 & 25 mM imidazole respectively. Finally eluted the protein with 5-10 ml of elution buffer and store at -80⁰C. Washed the column sequentially with 3 volume of wash buffer (without imidazole) and lysis buffer respectively and stored the Ni-NTA agarose in equal volume of 30% ethanol at 4⁰C. The purified protein was pooled for dialysis. The protein concentration was estimated by Bradford method (1976) and purity of recombinant proteins was determined by SDS-PAGE (Laemmli, 1970).

3.8 Western Blotting

The purified protein was confirmed by Western blot analysis in which the membrane was incubated with anti 6x-His epitope tag mice (Thermofisher) at 1:1000 dilution. The secondary antibody HRPO conjugated Anti-Mouse IgG at 1:1000 dilution and immunosignal was detected by using DAB system.

3.9 Homology Modelling of Recombinant buBSP1 Protein

The homology model of rec-buBSP1 protein was built with the Modeller version 9.23 program. For searching of suitable templates having maximum identity and query coverage for rBSP1 protein were identified by homology search using protein BLAST (Basic Local Alignment Search Tool) tool against PDB (Protein Data Bank) database. The structure of seminal plasma protein PDC-109 (*Bos taurus*) with PDB ID: 1H8P, Chain A and structure of KDA type IV collagenase (*Homo sapiens*) with PDB ID: 1EAK, Chain A were selected as templates for three dimensional (3D) modelling of the rBSP1 protein sequence. Modeller 9.23 facilitated in the development of 50 raw models of which the models with the lowest DOPE (Discrete) score was selected. The rBSP1

protein structure was further validated by PROCHECK (Laskowski *et al.* 1993) and ProSa web servers (Wiederstein M and Sippl MJ. 2005). For correctness and accuracy of 3D modeled of rBSP1 protein, 100 & 25 ns MD simulation was performed using OPLS-AA/L all-atom force field in Gromacs v software.

3.10 Prediction of Binding Site for Recombinant buBSP1 Protein-

The binding site of rec-buBSP1 protein is not available. Therefore, we predicted the binding cavity of the recombinant protein through Metapocket2 web-server (<https://www.h-its.org/de/software/metapocket2/>).

3.11 Selection and Preparation of Ligands

The two-dimensional (2D) chemical structures of heparin and PC were retrieved from PubChem and ChEBI database because of their allosteric property and functional similarity with small molecules structure. Further, the 2D structures were converted to a three-dimensional (3D) structures using OpenBabel program (O'Boyle) for performing molecular docking with rec-buBSP1 protein.

3.12 Molecular Docking

The molecular docking was performed and prepared a pdbqt file of rec-buBSP1 protein by using AutoDock 4.2.6 tool. The polar hydrogen and Kollman charges were added to the structure of rBSP1. Geister partial charges, non-polar hydrogen, and rotatable bonds were applied for both ligands. The structure of receptor and ligands were saved in "pdbqt" format for docking calculations. The 3D grid box with dimensions $X = 70\text{\AA}$, $Y = 70\text{\AA}$, and $Z = 70\text{\AA}$ with grid-point center $X = 40.044\text{\AA}$, $Y = 18.668\text{\AA}$, and $Z = 25.432\text{\AA}$ and the grid spacing of $0.375\text{\AA}$ were made to cover the binding cavity of rBSP1. The Lamarckian genetic algorithm (LGA) was employed to perform molecular docking in AutoDock. During the docking process, a maximum of 50 conformers was considered for each ligand which was set to terminate after 2500000 energy evaluations and the best docking conformation of rec-buBSP1 and ligands were selected and visually analyzed using Pymol (<https://pymol.org/2/>).

3.13 MD simulation of recombinant protein (rec-buBSP1) and Selected Ligands

The selected best binding mode of rBSP1 and ligand complexes were subjected to molecular dynamics simulation by GROMACS v 5.0.7 package. MD simulations were performed using Amber99B-ILDN force field. The heparin and PC topologies files were

generated using PRODRAG web server (<http://prodrgr1.dyndns.org/submit.html>). The protein-ligand complexes were solvated with TIP3P water model and a cubic box setting was done with a distance of 10 Å between the edge of the box and protein surface. Further neutralization of the protein-ligands complex by ions (Na⁺/Cl⁻) with 0.15M ionic strength and energy minimization was done using steepest descent algorithm. After energy minimization, both complexes were subjected to equilibration using NVT (constant number of atoms, volume, and temperature) and NPT (constant number of atoms, pressure, and temperature) ensemble for 1ns (nano-second). Finally, MD simulation of both complexes were run for 25 ns at a constant temperature (V-rescale method) and pressure (Parrinello-Rahman method) for trajectory analysis. The computation of the secondary structure of both complexes were carried out using DSSP v 2.0 (https://swift.cmbi.umcn.nl/gv/dssp/DSSP_1.html). Then binding free energy of both complexes were calculated using g_mmpbsa (Molecular Mechanics Poisson-Boltzmann Surface Area) gromacs tool (<https://bio.tools/gromacs>) and 2D graphs were plotted using Xmgrace (Grace5.1.21;<http://plasma-gate.weizmann.ac.il/Grace/>).

Methods: Objective 3: Effect of recombinant buffalo BSP on *in vitro* fertilization

3.14 Effect of recombinant buBSP1 on *in vitro* capacitation study

3.14.1 Preparation of sperm & assessment of semen quality

Semen samples were collected from Animal Breeding Research Center (ABRC) NDRI, Karnal and the samples having concentration @ 300 million spermatozoa/ml were used to evaluate progressive motility (>80%); total abnormalities (<10%) in this experiments (Chemineau, et al.1991). Around 0.250 ml semen was transferred to 10 ml of capacitation medium (BO) supplemented with different concentration of recombinant BSP proteins (20, 50, 100 µg/ml) and semen without treatment with protein was used as a control and incubated in CO₂ incubator (5% CO₂) at 37°C for 1-4 h to assess progressive motility, hyperactivity and acrosome reaction.

3.14.2 Evaluation of sperm motility percentage

Swim up technique was carried out in 15 ml centrifuge tubes, kept at a 45° angle and incubated at 37°C CO₂ incubator and determined individual motility percentage and acrosome reaction (AR %) after 1-4 h post-incubation. 100 µl of the sperm aliquots

placed on clean glass slides covered by a cover slip and observed at 40X in phase contrast microscope (40X) with a heated stage (37°C). Same sample was analyzed using CASA (computer assisted sperm analysis) for motility and functional spermatozoa percentage. Only progressive forward motility was screened for experiment among different treatments and control and determined the functional spermatozoa percentages by calculating the percentage of sperm cells with flagella beating & circular movement and was expressed by “pluses” (+ means HA% 20–40%, ++ means HA% 40–60%, +++ means HA% 60– 80% & ++++ means HA% >80%).

3.14.3 Checking of acrosome reaction status.

Then evaluated sperm acrosome status by Hoechst 33342 staining in which spermatozoa treated with different concentration of rec-BSP1 and control (without treatment) as mentioned above and both spermatozoa and 2mg/ml Hoechst 33342 staining solution were incubated at room temperature for 15 min then washed with PBS for several times. Then 100µl sperm suspensions spreaded over microscope glass slides & kept at room temperature to dry. The fixation was done in 70% and 95% ethyl alcohol respectively for 2 min then stained with FITC (@ 40mg/ml) and kept for 20 min. Then rinsed slide with distilled water 3-4 times and dried at room temperature and observed under oil immersion lens (100X) using Olympus BX50 light microscope to evaluate the proportion of acrosome status by analysis of morphology of spermatozoa and spermatozoa with an intact plasma and outer acrosomal membrane and fenestrations, vesiculation and loosening between plasma membrane and outer acrosomal membrane

3.15 In Vitro Fertilization study

3.15.1 Collection of Ovaries & processing for aspiration

Ovaries were collected from slaughter house (Gazipur, Delhi) and processed within 6 hours as per following procedure,

Procedure

1. Ovaries were washed 3 times with pre warmed NSS solution treatment with antibiotics and transferred to thermos flask.
2. Extra tissue surrounding the ovaries were removed by trimming and washed properly by NSS containing antibiotic.

Materials and methods

3. The ovaries having good number of follicles (2-8mm diameter) were selected for aspiration with a 19-gauge needle to a 10ml syringe containing the aspiration medium (TCM-199+0.3% BSA+50 µg/ml gentamycin sulfate)
4. The syringe containing aspirated oocyte, follicular fluid, granulosa cells were collected in 100mm x 100mm square Petri dishes with 13 mm grid.
5. Oocytes were searched at 20X under a zoom Stereomicroscope.
6. Oocytes were transferred to 35 mm dishes containing washing medium (TCM-199+10 % FBS+0.81mM sodium pyruvate+50 µg/ml gentamycin sulfate) and selected the Grade-A/B Oocytes having compact cumulus oocyte complexes with cumulus mass having ≥ 2 layer of cumulus cells and homogenous, granular ooplasm for *In Vitro Maturation* (IVM).

3.15.2 In Vitro Maturation of oocytes

The immature oocytes collected from developing follicles (antral or tertiary) were subjected to maturation in presence of exogenous hormonal supplement.

1. The oocytes were washed 3-4 times with washing medium (TCM-199+10 % FBS+0.68mM L-glutamine +0.81mM sodium pyruvate+50 µg/ml gentamycin sulfate)
2. Then the oocytes were washed thrice with IVM medium (TCM-199+10 % FBS+5µg/ml pFSH +1µg/ml estradiol-17 β +0.81mM sodium pyruvate+50 µg/ml gentamycin sulfate)
3. COCs were cultured in groups of 15-20 in 100 µl droplets of the IVM medium and overlaid with sterile mineral oil in Petri dishes for 24 h in a humidified CO₂ incubator (5% CO₂, 95% RH) at 38.5⁰C.
4. The maturation oocytes having cumulus cells expansion surrounding the zona pellucida about 2-4 times were selected for *In Vitro Fertilization* (IVF).

3.15.3 In Vitro Fertilization

The mature oocytes were co-incubated with processed frozen thawed capacitated semen in a Petri dish under controlled condition for union of male and female gametes, result in formation of pronuclei and zygote.

3.15.3a Sperm preparation & treatment with recombinant BSP (rec-BSP)

- ✓ Two straws of buffalo frozen semen straw were taken and thawed in pre warmed water (37⁰C) for 30sec.
- ✓ Semen straws were cut and contents are taken in 15ml centrifuge tube containing 10ml washing Bracket & Oliphant (BO) medium supplemented with 137 µg/ml sodium pyruvate & 1.942 mg/ml caffeine sodium benzoate.
- ✓ Centrifuged the above mixture at 1200 rpm for 7 min & discarded the supernatant and added the BO medium containing 10mg/ml fatty acid free BSA and centrifuged again at 1200 rpm for 7min.
- ✓ The pellet was suspended in 1ml of the capacitation and fertilization BO medium supplemented with 20, 50 and 100µg/ml recombinant buBSP1, 10 µg/ml heparin (control) and leave for 10min for swim.

3.15.3b Washing of IVM Oocytes

- ✓ The in vitro matured oocytes were washed thrice with the capacitation and fertilization BO medium.
- ✓ Then transferred 15-20 oocytes to 100µl droplets capacitation and fertilization BO medium.

3.15.3c Co-incubation of Oocytes and Spermatozoa

50µl of processed spermatozoa (2-4 million spermatozoa/ml) were added to the droplets containing oocytes and covered with sterile mineral oil and incubated for 16-18 hours in a humidified CO₂ incubator (5% CO₂, 95% RH) at 38.5⁰C for *In Vitro Fertilization*.

3.15.4 In Vitro cultures of embryos

- ✓ The presumptive zygotes were cultured in serum free system supplemented with different growth factors such as insulin like growth factor (IGF-1) to improve the survival of embryos serves as anti-apoptotic and protects from heat shock factors, epidermal growth factor (EGF) having positive effect on blastocyst number cell number and stem cell factor (SCF) to enhance the development of embryo into late blastocyst and hatching stage. Similarly, cysteamine, melatonin, ascorbic

Materials and methods

acid, mercapto ethanol and glutathione etc. protect embryos from oxidative stress.

- ✓ The cleavage of fertilized oocytes and their further development into blastocysts is an indication of the efficiency of an in vitro embryo production. At the end of sperm –oocyte incubation, cumulus cells were washed off by gentle pipetting. Discarded the TCM-199 from IVM droplets and washed the oocytes with 3 times with modified Charles Rosenkrans medium with amino acids (mCR_{2aa}) containing 0.6% BSA.
- ✓ Presumptive zygotes were cultured in 100µl droplets of mCR_{2aa} on original beds of granulosa cells for 48hours in a humidified CO₂ incubator (5% CO₂, 95% RH) at 38.5⁰C. Embryos were shifted to the IVC medium (mCR_{2aa} + 0.06% BSA + 10%FBS) cultured up to 8 days' post insemination in a humidified CO₂ incubator (5% CO₂, 95% RH) at 38.5⁰C. Medium was replaced with 50% of fresh IVC medium every 48h.

3.15.5 Analysis statically

The effect of recombinant BSP on *In Vitro Embryo development* was analyzed by calculating the cleavage and blastocyst rate percentage using biostatistical tool. Compared the percentage of embryo developed in various embryonic stages at different days 5-7 post insemination between treatment groups & controls.

3.15.6 Counting of Blastocysts

Counting the total 1 numbers day 9 blastocysts cells was done after Hoechst 33342 staining of 20-25 IVF blastocysts nuclei @ 10 ng/ml for 10 minutes and washed three times in Dulbecco's PBS. The stained (Hoechst 33342) embryos were mounted individually on microscopic slides with a cover slip in glycerol and examined the mounted preparation under UV.

CHAPTER -4

Results

RESULTS

4.1 Extraction of Total RNA from seminal vesicle and Synthesis of cDNA

The total RNA was extracted by Trizol method. RNA samples were further checked on 1.5% denatured agarose gel, revealing three distinct bands corresponding to 5.5, 18 and 28S rRNA (Fig.4.1), For quantitative and qualitative analysis, RNA samples were visualized on nanodrop spectrophotometer (Table 4.1)

Table 4.1 Checking of RNA concentration by spectrophotometry

Sample ID	260/230	260/280	Concentration (ng/μl)
1	0.87	1.93	1230
2	1.04	1.93	951.5
3	1.49	1.98	860.1

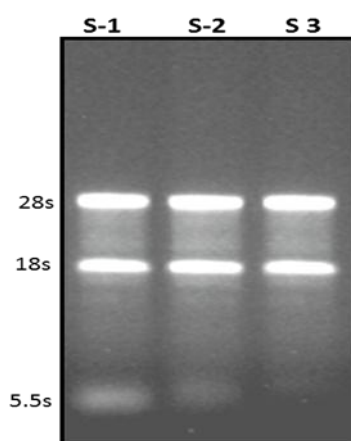


Figure 4.1: RNA samples were further visualized on 1.5% denatured agarose gel showed 5.5S,18S & 28S subunit

Synthesis of cDNA was carried out using pelleted RNA using random hexamer primer and M-MuLV reverse transcriptase in a typical 20 μl reaction mixture. After the cDNA synthesis, it was stored at -20°C for further use.

4.2 Amplification of buffalo Binder of sperm protein (buBSP) Gene

buBSP gene was amplified using gene specific primer sets as described in

Results

Section Table-3.1. The amplicon was run on 2 % agarose gel and three bands of different size (approx. 0.5Kb, 0.35Kb, 0.25Kb) were observed (Fig. 4.2). The band with ~0.5kb size gene was used for further study.

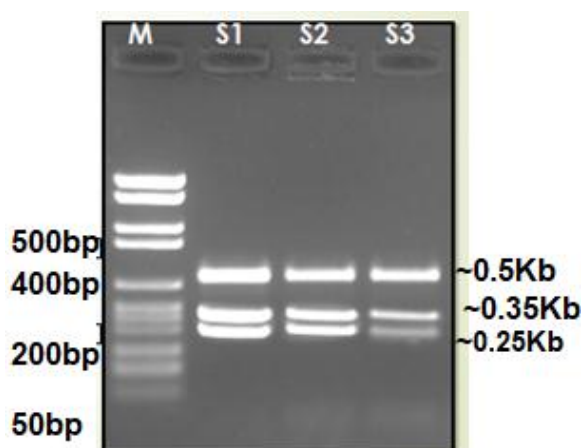


Figure 4.2: Agarose Gel Electrophoresis of PCR Amplified BSP Product; Lane M: 50bp DNA Ladder; Lane S1, S2&S3: BSP

Four such reactions of 25 µl each was set and the product was pooled for purification using Qiagen gel extraction kit. The amplicon was run on 2 % agarose gel to check purity of amplicon.

4.3 Cloning of BSP1 PCR Product in pEASY blunt cloning Vector

Purified BSP PCR product was cloned in cloning vector keeping the insert: vector ratio to 3:1.

$$\begin{aligned} \text{ng of insert (PCR product)} &= \frac{\text{ng of vector} \times \text{kb size of insert} \times 3}{1\text{kb size of vector}} \\ &= \frac{55 \times 2.1 \times 3}{2.9} \\ \text{ng of insert (PCR product)} &= 120 \text{ ng} \end{aligned}$$

By using the formula, 120 ng of purified PCR product was ligated to 55 ng of vector. The competent *E. coli* DH5α cells were successfully transformed. The recombinant clones were first screened by visualizing its blue-white colour from LB/ampicillin/ X-gal/ IPTG plates (Fig.4.3). Nine such colonies were picked up for colony PCR, out of which seven were found positive (Fig. 4.4).



Figure 4.3: Transformed Colonies Appeared on (+) Ampicillin LB Plates. White Colonies were recombinants While Blue Colonies were Non-recombinants



Figure 4.4: Colony PCR of BSP gene where Lane M: DNA Ladder; Lane 1-7: Positive clones & Lane 8,9: negative clones

Plasmids were isolated from positive recombinant clones grown overnight by alkaline lysis method and were sent for custom sequencing of the cloned amplicon.

4.4 Sequence Analysis

plasmids isolated from ten recombinant clones and were custom sequenced using different sets of primer (T7 universal, and gene specific reverse primer). The forward and reverse reads were aligned to remove any sequencing error. The consensus sequences derived from the forward and reverse reads were then aligned by ClustalW2 program for multiple sequence alignment by using nucleotide and protein sequence of different

Results

isoforms of BSP from the available database at NCBI. The translated sequences were highly identical to the sequences present in other species namely mouse, human and Bos Taurus. It showed seven cysteine, five tyrosine, four tryptophan, four phenylalanine, two lysine, two proline, two serine, two leucine, two aspartates, one methionine, one glycine, and one asparagine residues which were conserved in all the proteins sequence of different isoforms of BSP. The changes in the nucleotides and amino acids located at different position in various isoforms is shown in fig demonstrated (Fig. 4.5a, b). The similar sequences of different species to predominant isoform BSP sequence were retrieved from UniProt database and aligned by muscle program using UPGMA method. MegavX software. was used to reconstruct the phylogenetic tree by maximum likelihood (ML) method and JTT model with 1000 bootstrap replicates (Fig 4.6). It was shown in the phylogenetic tree that the predominant isoform of buffalo BSP is closely related to bovine seminal plasma protein PDC 109 (Isoform1). Phylogenetic analysis showed high similarity of the predominant sequence with cattle which suggests that the rec-buBSP1 gene is closely related to BSP sequence of other ruminants. However, BSP of human and mice made separate group.

BSP Isoform 3	ATGGCACCGCTAGTGGGGCTCTTCTGATTGGGCTGGTGCCTCTGTGTTTCAACAATA	60
BSP Isoform 1'	ATGGCCTTGCACTTGGGTCTTTCTGATTGGGCCGAGTAAGCGTGTGTTTGCAGTTA	60
BSP Isoform 1	ATGGCACTGCAGTTGGGGCTCTTCTCATTTGGGCTGGCGTGTCTGTGTTTCTACAATG	60
BSP Isoform 2	ATGGCACTGCGTTTGGGGCTCTTCTGATTGGGCTGGCGTGTCTATGTTTCTACAATG	60
	****, ** ***** ** * ***** ** * .: ***** :**, *	
BSP Isoform 3	CACCCTGTGAATGGAGGGGATATCCCGACCCAGGAGCAAACCCACGCCTCCTGGAATG	120
BSP Isoform 1'	GACCCTGTAAACGGAACCAGG-----	82
BSP Isoform 1	GACCCTGTGAATGGAGATCAGG-----	82
BSP Isoform 2	GACCCTGTGAATGGAGATGAGC-----	82
	*****, ** **... *	
BSP Isoform 3	GCAGATGAGCTGCCGACAGAGACGTATGACCTTCCTCCCGAAATTTACACTACTACATTT	180
BSP Isoform 1'	-----A-----TGAAGGGGTCTTTACAGAA-CTGACCCA--GG-----	112
BSP Isoform 1	-----A-----CGAAGGTGTTTCTACTG-AACCTACCCA-----	110
BSP Isoform 2	-----AGCTGTCTGAGGA---CAACGTCATTCTTCTAAAGA-----	116
	* ...* :: : ** *	
BSP Isoform 3	TTGCCTCGCACCATTATCCTCAAGAGGAGATGCCGTATGACGACAAACCTTTTCTAGC	240
BSP Isoform 1'	----ATCGCC-----CAGCCGAGC	127
BSP Isoform 1	----AG-A-----CGGTCCTGC	122
BSP Isoform 2	----AAAGAA-----AGACCCTGC	131
	.*, *****	

BSP Isoform 3 TTGTTGAGCAAAGCCAACGATCTTAATGCAGTTTTTGAAGGTCCTGCATGTGCTTTCCCA	300
BSP Isoform 1' TGCCGGAGGACAGCGTA-----CCAGATGAAGAAAGAGTATTCCCT	168
BSP Isoform 1 TG-----AATTACC-T-----GAAGACGAAGAATGCGTTTTCCCA	156
BSP Isoform 2 TTCTGGTGCAGAAAC-C-----AAAGATAATAAATGTGTTTTCCCA	171
* * .. .***.*.*.*****.	
BSP Isoform 3 TTCACCTATAAAGGCCAAAAAGTATTATATGTGCACGCGCAAAAATTCCGTATTACTATGG	360
BSP Isoform 1' TTCACGTATCGCAACCGTAAACATTTTGACTGTACACTTAGAGGTAGCATCTTCCCCTGG	228
BSP Isoform 1 TTCGTCTATAGAAACAGAAAGCATTTTGACTGCACAGTGCATGGTTTCCTTATCCCCGTGG	216
BSP Isoform 2 TTCATCTATGGAACAAAAAGTATTTTGACTGCACATTGCATGGTTTCCTTATTCCTGTGG	231
. *** * ..*.*..*.* ** * ..*.*.*.*.***	
BSP Isoform 3 TGTTCCTCGATACAGAATATCAAGGAACTGGAAATTCTGTACTGAGAGAGACGAACCC	420
BSP Isoform 1' TGTAGTCTGGATGCCAACTACGTGGGACGCTGGAAATACTGCGCGCAGCGTACTACGCC	288
BSP Isoform 1 TGTTCCTCGATGCAGACTATGTAGGAAGATGGAAATACTGTGCTCAGAGAGACTATGCT	276
BSP Isoform 2 TGTTCCTCGATGCAGACTATACAGGAAGATGGAAATACTGTACTAAGAATGACTATGCC	291
. ** ***.*.*.*..*****.*.*.*.*.*.*	
BSP Isoform 3 GAATGTGTCTTCCCCTTTATCTATCGCAAGAAATCCTATGAAAGTTGCACACGGGTGCAT	480
BSP Isoform 1' AAGTGTGTCTTCCCCTTTATTTACGGGGGTAAGAAATACGAGACCTGCACGAAGACGGG-	347
BSP Isoform 1 AAATGTGTCTTCCCCTTTATCTATGGAGGCAAGAAATATGAGACTTGCACAAAAATTGGG	336
BSP Isoform 2 AAATGTGTCTTCCCCTTTATCTATGAAGGCAAGTCATATGACACTTGCATAATAATTGGG	351
*.***** ** ** ** .. **.* ** ** ** ..	
BSP Isoform 3 AGTTTTTCTGGAGGAGATGGTGCTCACTCACTTCAAACCTATGACCGGGATAAAGCTTGG	540
BSP Isoform 1' --TTCAATGTGGATGTCTTGGTGCTCTTATCGCCCAACTACGACAAAGATAGAGCCTGG	405
BSP Isoform 1 AGTATGTGG---ATGCTTGGTGCTCACTCTCTCCAACTACGACAAGGACAGAGCTTGG	393
BSP Isoform 2 AGTACATTTATGAACTATTGGTGCTCACTCTCTTCAAACCTATGATGAGGATGGAGTTTGG	411
*. : * : *****.*.*.*.***** ** ..*.*.***	
BSP Isoform 3 AAGTATTGCTAG	552
BSP Isoform 1' AAGTATTGT---	414
BSP Isoform 1 AAGTATTGCTAG	405
BSP Isoform 2 AAGTATTGCTAG	423

Figure 4.5a: Multiple Alignments of nucleotide sequence of different isoforms of BSP. residues Identical to the sequences of indicated by dots (.), spaces or dash (-) denotes gap

Results

BSP Isoform 3 MAPLVGLFLIWAGASVFQQLHPVNGGDIPDPGSKPTPPGMADELPTETYDLPPEIYTTTF	60
BSP Isoform 2 MALRLGLFLIWAGVSMFLQLDPVNGDEQLSEDNVILPKEKKDPA-----	44
BSP Isoform 1b MALQLGLFLIWAGVSVFLQLDPVNGDQDEGVSTEPT-QDGPael-----	43
BSP Isoform 1a MALQLGLFLIWAGVSVFLQLDPVNGNQDEGVFTELT-QDRPAEL-----	43
** .*****.*.*.******. . .	
BSP Isoform 3 LPRTIYPQEEMPYDDKPFPSLLSKANDLNAVFE GPACAFPFYKGGKKYYMCTRKNsvllw	120
BSP Isoform 2 -----SGAETKDNKCVFPFIYGNKKYFDCTLHGSLFLW	77
BSP Isoform 1b -----PED---EECVFPFVYRNrkHFDCTVHGSLFPW	72
BSP Isoform 1a -----PEDSVPDEERVFPFTYRNrkHFDCTLRGSIFPW	76
.*** * .*:.*.*.*.*	
BSP Isoform 3 CSLDTEYQGNWKFCTERDEPECVFPFIYRKKSYESCTRVSFFWRRWCsLTSNYDRDKAW	180
BSP Isoform 2 CSLDADYtGRWKYCTKNDYAKCVFPFIYEGKSYDTCIIIGSTFMNYWCSLSSNYDEDGVW	137
BSP Isoform 1b CSLDADYVGRWKYCAQRDYAKCVFPFIYGGKKYETCTKIGSMWM-SWCsLSPNYDKDRAW	131
BSP Isoform 1a CSLDANYVGRWKYCAQRDYAKCVFPFIYGGKKYETCTKTGSMWM-SWCsLSPNYDKDRAW	135
*****.*.*.*.*.*.******.*.*.*.*.*.*.*	
BSP Isoform 3 KYC	183
BSP Isoform 2 KYC	140
BSP Isoform 1b KYC	134
BSP Isoform 1a KYC	138

Figure 4.5b: Multiple Alignments of protein sequence of different isoforms of BSP found in our study. residues identical to the sequences of indicated by dots (.). spaces or dash (-) denotes gap

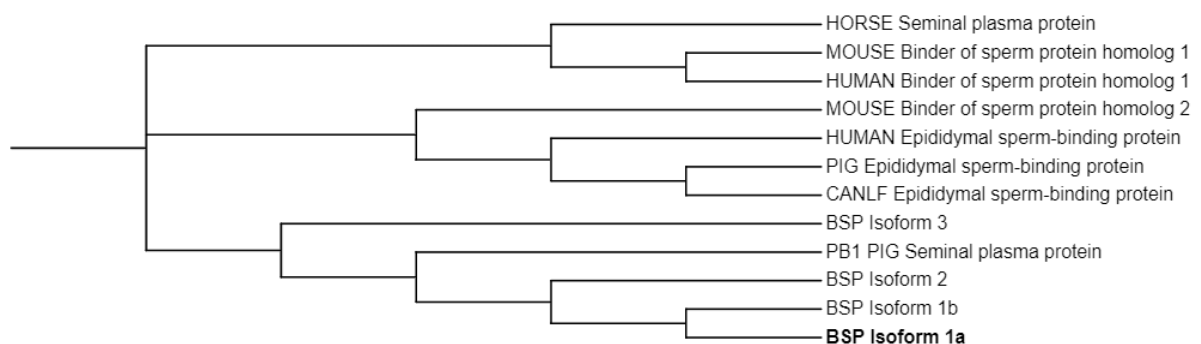


Figure 4.6: Schematic of Maximum Likelihood consensus tree with 1000 bootstraps (MegaX).

4.5 Expression of predominant BSP isoform in *E. coli* expression host using Prokaryotic Expression Vector (pET28a)

The relatively predominant isoform buBSP1 sequence was amplified by designed primers set with standardized annealing temperature of 52⁰ C and 468 bp was observed on 1% agarose gel (Fig.4.7). BSP1F-5'AGCTGGATCCATGGCACTGCAGTTGGGGCTC3' and BSP1R-5'CTAGCTCGAGGCAATACTTCCAAGCTCTGTCTT3'. The gene was cloned into pET28a vector containing sequences for T7 promotor/lac operator, NdeI /XhoI restriction site and a His-tag. Ligation of buBSP1 gene in recombinant vector pET228_buBSP1 was further confirmed by RE digestion and sequencing (Fig.4.8 a, b). In order to express predominant buBSP1 gene, both expression vectors and recombinant plasmid were digested with *NdeI* and *XhoI* restriction enzymes. Restriction enzymes digested vectors and recombinant plasmid were run on 1.5% agarose to purify the desired insert and vector from gel using Qiagen gel extraction kit. The purified vectors and inserts were ligated in order to produce recombinant plasmid keeping the 3:1 molar ratio of insert: plasmid. The recombinant plasmids were transformed in expression *E. Coli* strain BL21 (DE3).

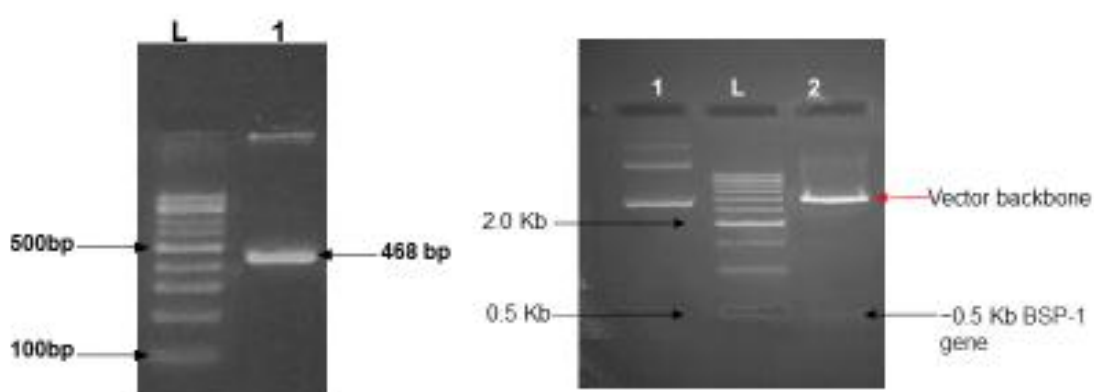


Figure 4.7: PCR amplicon (lane1) loaded on 2% agarose gel, L: DNA ladder

4.8a: Restriction enzyme digestion of NdeI/ XhoI loaded on 1.5% agarose gel

Results

5'CATATGGCCTTGCAGTTGGGTCTTTTCTTGATTGGGCCGGAGTAAGCGTG
TTTTTGCAGTTAGACCCTGTAAACGGGAACCAGGATGAAGGGGTCTTTACAG
AACTGACCCAGGATCGCCCAGCCGAGCTGCCGGAGGACAGCGTACCAGATG
AAGAAAGAGTATTCCCTTTCACGTATCGCAACCGTAAACATTTTGACTGTACA
CTTAGAGGTAGCATCTTCCCCTGGTGTAGTCTGGATGCCAACTACGTGGGACG
CTGGAAATACTGCGCGCAGCGTGACTACGCCAAGTGTGTCTTCCCTTTCATT
ACGGGGGTAAAGAAATACGAGACCTGCACGAAGACGGGTCAATGTGGATGTC
CTGGTGCTCCTTATCGCCCAACTACGACAAAGATAGAGCCTGGAAGTATTGT
CTCGAG CACCACCACCACCACCAC TGA 3'

Figure 4.8b: Sequence of expression construct (rec-buBSP1) intact ORF with His tag-Black Highlight-RE sites (NdeI/XhoI), Blue Highlight-rec-BSP1 gene, Red Highlight-His tag & stop codon

4.6 Expression of recombinant buffalo BSP1 (rec-buBSP1)

PCR positive clone was inoculated for big batch expression (200ml) & protein purification (starter) and grown till 0.6 OD and induced with 1mM IPTG at 37°C for overnight. Before and after induction (4 & 16 h) samples were analyzed on SDS-PAGE (Fig. 4.9a) which showed expression of high intensity band protein in the region of ~17 kDa protein. Further the E coli cells were induced at low temperature 16°C, the proportion of soluble fraction increased which was confirmed by SDS PAGE (Fig.4.9b).

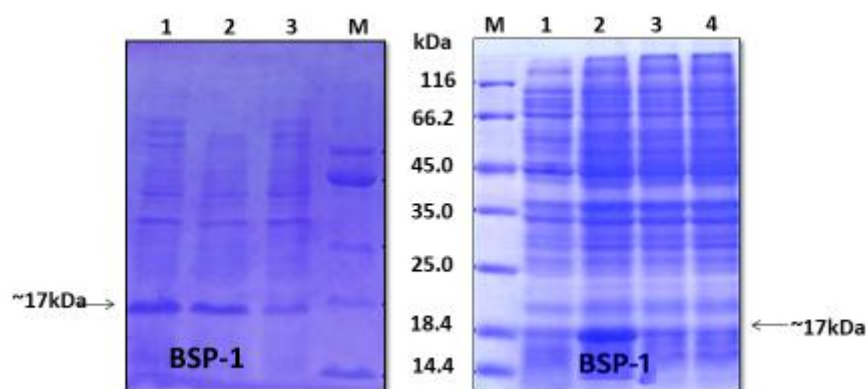


Figure 4.9: a. Induction of PCR Positive Clones with 1mM IPTG at 37°C. Lane 1: 16 hours after induction sample (whole cell extract), Lane2: 4 hours after induction sample (whole cell extract), Lane3: Before induction sample (whole cell extract) showing slight leaky expression, Lane M: Protein Marker

b. Induction of PCR Positive Clones with 1mM IPTG at 16°C. Lane M: Marker (Unstained); Lane 1: Un-induced Clone (pET28a); Lane 2-4: Induced clone (pET28a)

4.7 Purification of rec-BSP1 Protein by Ni-NTA Agarose

The recombinant proteins contained 6x His tag fused to N-terminal of proteins. The recombinant proteins were eluted from Ni-NTA agarose column. The culture was pelleted down and pellet suspended in Affinity column-lysis buffer which were then subjected to sonication and centrifuged after sonication at 10,000 rpm for 15 min at 4°C. the supernatant obtained after Sonication and pellet were loaded on 15% SDS-PAGE for visualization of protein (Fig 4.10). Eluted fractions were pooled and further purified by dialysis (Fig4.11). The western blot analysis was done by using anti his mouse antibody and HRP conjugated secondary antibody with DAB system as a substrate. The purified buBSP-1 protein was observed as single band of ~17kDa size (fig.4.12). The quantification of rec-buBSP1 concentration was done by Bradford assay.

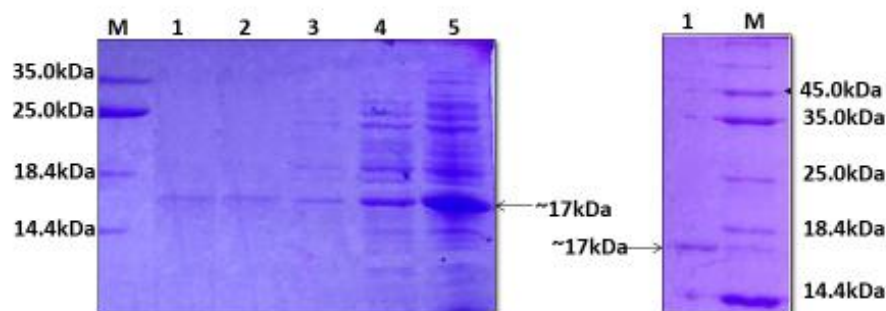


Figure 4.10: Pellet and Supernatants after sonication were loaded on 15% SDS-PAGE where Lane 1: 1st sonication pellet, Lane 2: 2nd sonication pellet, Lane 3: 3rd sonication pellet, Lane 4: 4th sonication pellet, Lane 5: 4th sonication supernatant M: Marker

Figure 4.11: Dialyzed pooled elutes loaded on 15% SDS-PAGE where Lane 1: Dialyzed pooled elutes; M: Marker

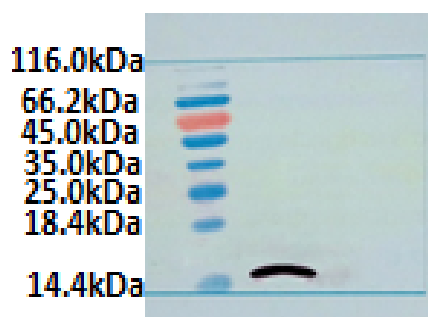


Figure 4.12: Western Blot analysis of rec-buBSP1 using anti his mouse Ab, HRP conjugate 2^o Ab showed ~17 kDa size protein for confirmation

4.8 Three Dimensional Modeling of rec-buBSP1 and Structure Validation

Modeller v9.23 software was used to predict the tertiary structure of 138 amino acids long rBSP1 protein sequence. The suitable templates were used to predict the tertiary structure of rBSP1 by performing a homology search using BLASTp against the PDB database. In search of BLASTp two templates such as seminal plasma protein PDC-109 (*Bos taurus*), PDB ID: 1H8P, Chain A, with a query coverage of 81% and sequence identity of 86.73%. and the structure of KDA type IV collagenase (*Homo sapiens*), PDB ID: 1EAK, Chain A, with a query coverage of 60 % and sequence identity of 34.69% were considered for 3D modeling of rBSP1 protein sequence. Modeller 9.23 software was used to develop 50 raw models. Among which the model with the lowest DOPE score was taken for further study. The Ramachandran plot analysis was verified generated protein structure through different protein structure validation web servers PROCHECK (Table 4.2) and ProSA. The Z-score (-3.91) for rBSP1 was calculated by Pro-SA web which is within the range of experimental structure (Fig. 4.13A) and validation of the secondary structure of rBSP1 was carried out by STRIDE software (Fig. 4.13B). For correctness and accuracy of 3D modeled of rec-BSP1 protein, 100 ns MD simulation was performed using OPLS-AA/L all-atom force field in Gromacs software (Fig.4.13 C, D, E, F, G). The dynamic stability of the rBSP1, RMSD value in order to check the stability of the complexes and it was shown that after a period of 10-ns indicating stable conformation of the modelled protein.

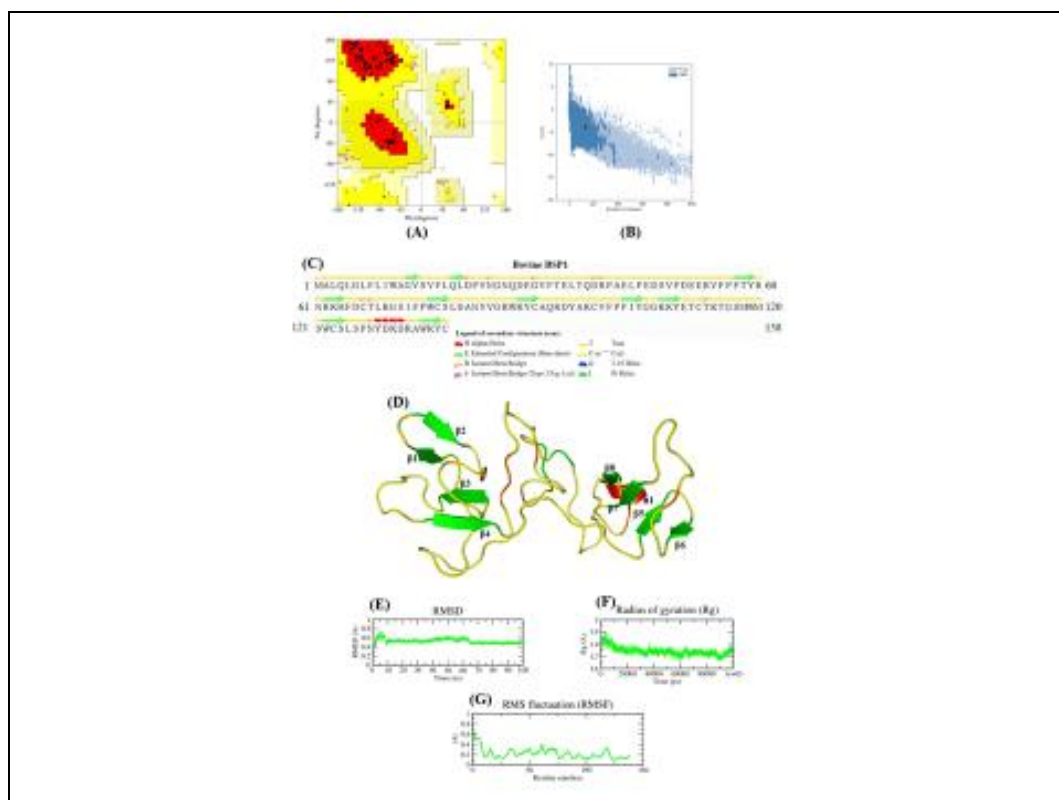


Fig. 4.13: Structural model of rec-BSP1 protein. A, Overall quality of rec-BSP1 protein structure validation by using procheck for Ramachandran plot. B, rec-BSP1 protein structure validation using Pro-SA web server. C, Secondary structure prediction of rec-BSP1. D, 3D structure of rec-BSP1 protein, the image was generated using PyMOL, where green color represent the Beta-Sheet and red color represent the Coil. E, graphical representation of RMSD analysis of 100-ns simulation. F, Radius gyration of 100-ns simulation. G, RMSF analysis of 138 amino acid residues of rec-BSP1.

Table 4.2: PROCHECK (Ramachandran plot) analysis of various model validation scores of the modeled rec-BSP1

Plot statistics		
Residues in most favoured regions [A,B,L]	111	93.3%
Residues in additional allowed regions [a,b,l,p]	6	5.0%
Residues in generously allowed regions [-a,-b,-l,-p]	2	1.7%
Residues in disallowed regions	0	0.0%

Number of non-glycine and non-proline residues	119	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	9	
Number of proline residues	8	

Total number of residues	138	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

4.9 Molecular Docking Analysis

Then two ligands were docked to rBSP1 using AutoDock 4.2.6 tool. The polar hydrogen and Kollman charges were added to the structure of rBSP1. Geister partial charges, non-polar hydrogen, and rotatable bonds were applied for both ligands. In AutoDock tool, molecular docking was performed using the same grid box ($X = Y = Z = 70 \text{ \AA}$) and grid points center ($X = 40.044 \text{ \AA}$, $Y = 18.668 \text{ \AA}$, $Z = 25.432 \text{ \AA}$) and the grid spacing of $0.375 \text{ \AA}$ were made to cover the binding cavity of rBSP1. The Lamarckian genetic algorithm (LGA) was employed to perform molecular docking in AutoDock. The docking score was determined based on their binding energy and ligand efficiency score and number of hydrogen bonds formed. The fifty docking conformation of each ligand was docked to the binding site of the rBSP1 which was set to terminate after 2.5×10^6 energy evaluations. After docking, the lowest binding energy conformations of each molecule were taken for further study. Summary of the highest binding energy, ligand efficiency and number of hydrogen bonds formed of the ligands is presented (Table 4.3). The binding site of rBSP1 with heparin and PC interacting residues are mentioned (Fig. 4.14 A, B). Further, MD simulations were performed to understand its dynamics stability

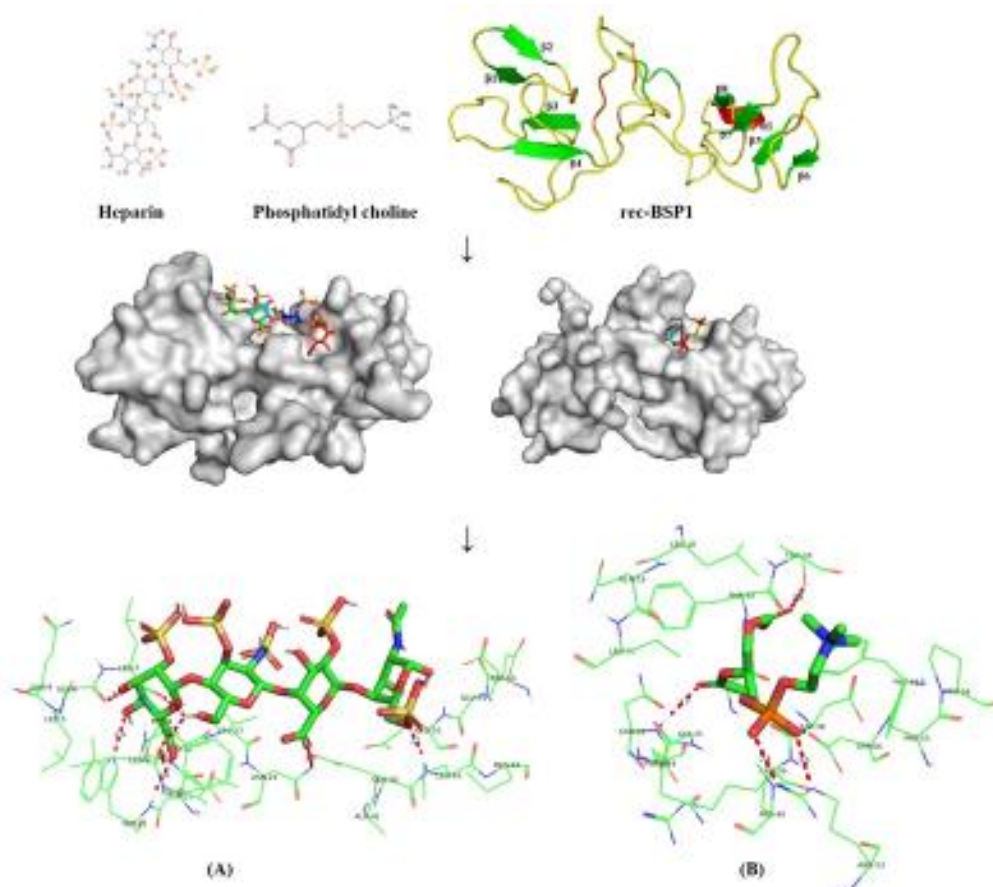


Figure 4.14: Docking pose of Protein-ligand complex. The structural representation the binding site of heparin and PC docked complex with interaction represented in red dotted lines. A, Heparin docked complex. B, PC docked complex.

Table.4.3: Binding energy, Ligand efficiency and Hydrogen bond formation of rBSP1 protein.

SN	Compounds	Binding Energy (kcal/mol)	Ligand Efficiency	Hydrogen bond	
				Residues	Bonds
1	Heparin	-5.44	-0.25	ASN 26 THR 33 ARG 53 ASP 66	4
2	Phosphatidyl Choline	-5.21	-0.07	THR 33 GLU 34	2

4.10 MD Simulation of Heparin and Phosphatidyl Choline Docked Complexes

MD simulation study was performed by GROMACS v 5.0.7 package under Amber99B-ILDN force field for the two complex systems to understand the dynamics and structural behavior of the rBSP1 when attached with heparin and PC. Topologies files were generated using PRODRAG web server. The protein-ligand complexes were solvated with TIP3P water model and a cubic box setting was done with a distance of 10 Å between the edge of the box and protein surface. Neutralization of the protein-ligands complex by ions (Na^+/Cl^-) with 0.15M ionic strength and energy minimization was performed using steepest descent algorithm. After energy minimization, both complexes were subjected to equilibration using NVT (constant number of atoms, volume, and temperature) and NPT (constant number of atoms, pressure, and temperature) ensemble for 1ns (nana-second). Finally, MD simulation of both complexes were run for 25 ns at a constant temperature (V-rescale method) and pressure (Parrinello-Rahman method) for trajectory analysis. The secondary structure predictions of both complexes were carried out using DSSP v 2.0. Then binding free energy of both complexes were calculated using *g_mmpbsa* tool and 2D graphs were plotted using MATLAB. Then root-mean-square deviation (RMSD), Radius of gyration (RG) Residue-based Root Mean Square Fluctuations (RMSF) and Solvent accessible surface area (SASA) were used for investigation of dynamic stability of the complexes. The RMSD graph was plotted to measure the difference between backbone of protein from initial to final structural conformation and assessed the stability of protein-ligand complex during course of simulation. MD simulation was done to calculate the RMSD value in order to check the stability of the complexes and it was shown that average deviation of heparin and PC complexes is approximately 0.43 and 0.89 nm. It is indicating that heparin is more stable than PC complex (Fig. 4.15A). Similarly, RMSF was carried out to understand the flexibility and superimposed graphical representation of each residue. The RMSF value was calculated the magnitude of fluctuation of each residue and it was shown that the first complexes with heparin having fluctuation range of from approximately 0.4–0.7 nm first 30 residues after that it showed average fluctuation of 0.4 nm. Compared to heparine complex, the plots for PC Complex contain more noises and larger RMSF values, which indicate relatively a weak binding between the target and the substrates The binding site residues predicted by Metapocket server were all found to have less fluctuation for the recombinant protein-heparin complex compared to recombinant protein-PC complex

indicating intactness and rigidity of the binding cavity (Fig. 4.15B). Radius of gyration value was calculated the compactness and structural changes of complex by *gmx gyrate*. It was observed that average Rg value of 1.93 nm over the simulation period of 25 ns in the recombinant protein-ligands complexes. The difference between the recombinant protein-heparin complex relatively more stable than recombinant protein-PC complex after a period of 7 ns indicating stable folding of the modelled protein (Fig. 4.15C). Calculation and analysis of SASA was done for recombinant protein with heparin and PC complexes to understand their interaction and solvent accessibility by using *gmx sasa*. The average sasa value for heparin complex was 112, whereas PC complex 107 nm² over a period of 25ns. It was shown that recombinant protein-heparin complex has less fluctuation than recombinant protein-PC complex (Fig. 4.15D). The analysis of hydrogen bond between rBSP1 and the ligands were calculated for overall stability of protein-ligand complexes using *gmx hbond* over the period of 25 ns simulation. The binding site was analyzed for hydrogen bonding with ligands and it was shown that the rBSP1-heparin complex having higher numbers of H-bond (~2.229) compared to rBSP1-PC complex (~0.638) (Fig. 4.16). It was indicated that rBSP1-heparin complex was more stable interacting complex. The MD trajectories result files were used for Principal component analysis (PCA) for determination of conformational changes w.r.t recombinant protein function. The eigenvalues were calculated the movement of atoms meant for conformational changes which were obtained diagonalizing the covariance matrix of the C α atomic fluctuations against the equivalent Eigenvectors indices. It was shown that the projection of trajectory along PC1 and PC2 and recombinant heparin a higher value of trace of covariance matrix than PC indicating higher flexibility and increased collective motion. Protein-heparin complex showed the least conformational changes due to decreased collective motions than PC system. Determination of the PCA and RMSF investigations evoked similar changes about structural fluctuations of the protein-substrate complexes (Fig. 4.17A, B). Definition of Secondary Structure of Protein (DSSP) algorithm was applied to check the secondary structure changes during MD simulation. It was observed that there were significant structural changes in recombinant protein (rBSP1) which indicated the increase in helical content and absence in β -sheet (Fig. 4.18).

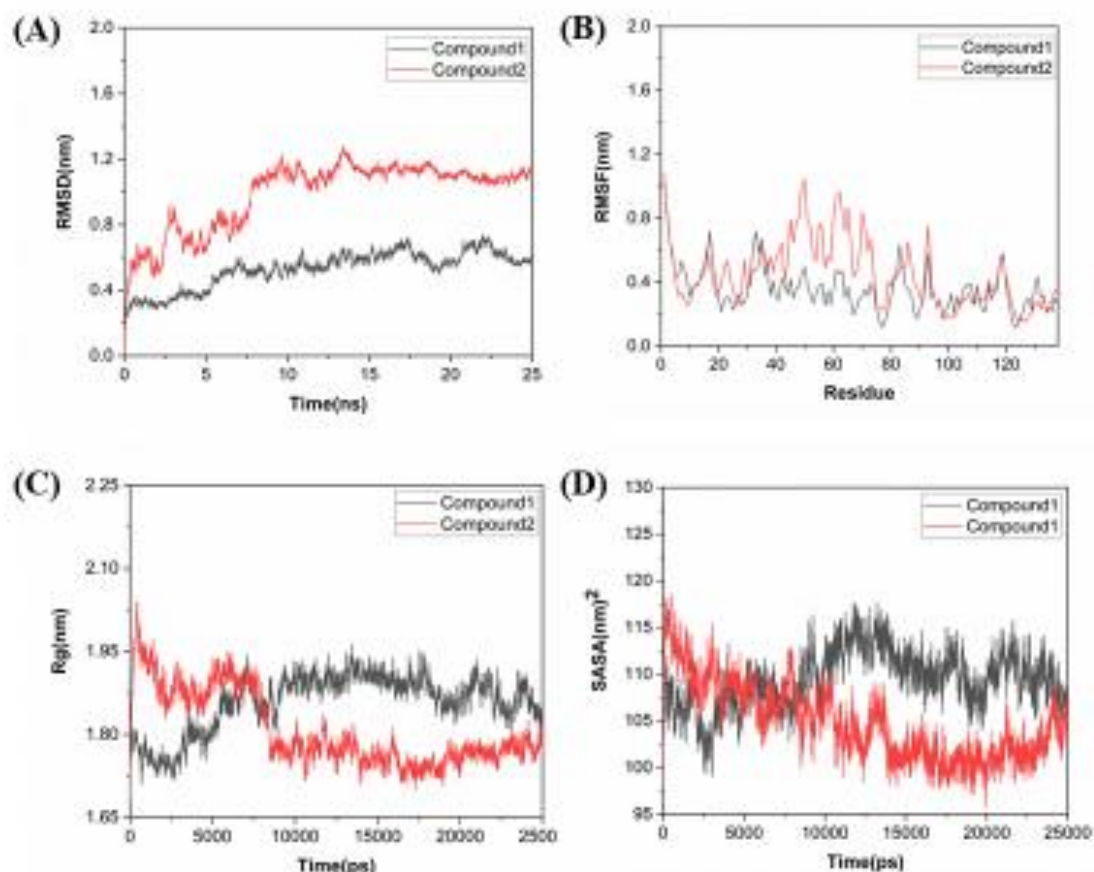


Figure 4.15: Dynamics stability of rec-BSP1 with heparin and PC complexes during 25-ns MD simulation. RMSD analysis of ligand Compound1 (heparin, black), and Compound2 (PC, red) protein backbone conformation. B, RMSF analysis of 138 amino acid residues is represented for Compound1 (heparin, black) and Compound2 (PC, red) protein complexes. C, Radius gyration of Compound1 (heparin, black) and Compound2 (PC, red). D, Solvent accessible surface area (SASA) of Compound1 (heparin, black) and Compound2 (PC, red).

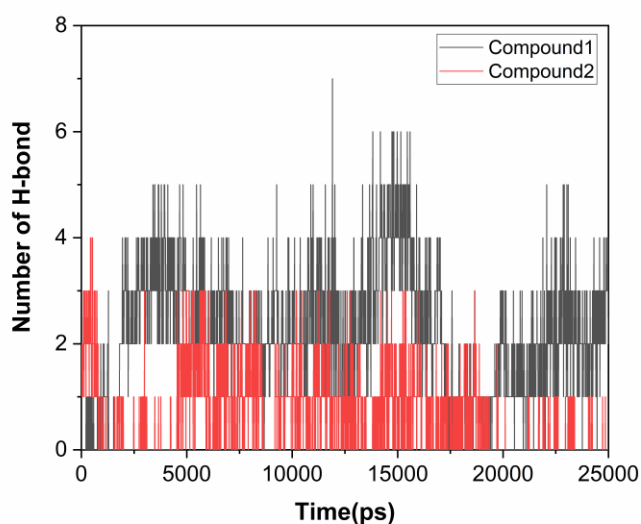


Figure 4.16: Hydrogen bond formation by rec-BSP1 protein with Compound 1 (heparin, black) and Compound2 (PC, red) during 25-ns simulation.

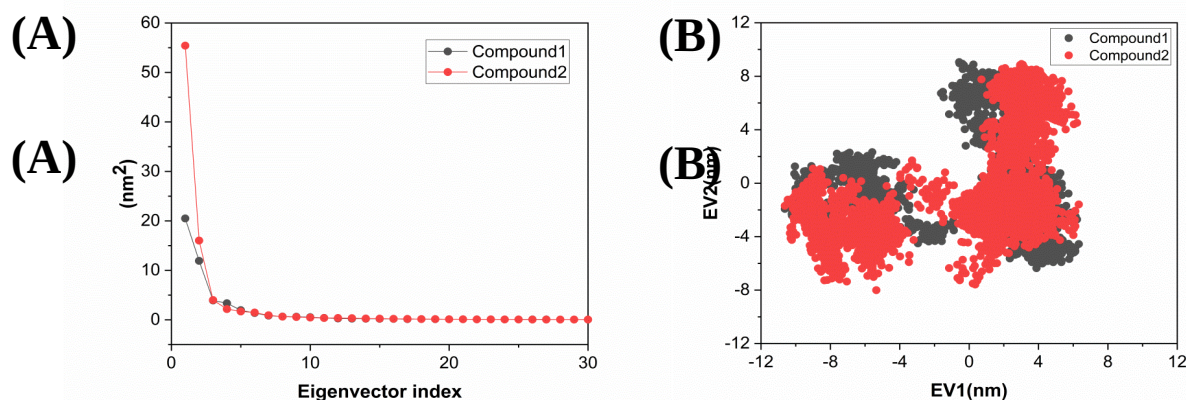


Figure 4.17: Principal component analysis of rec-BSP1 with heparin and PC complexes. A, The plot displaying the eigenvalue of both Compound 1 (heparin, black) and Compound2 (PC, red) of the first 30 eigenvectors. (B), The 2D Projection motion of the Compound 1 (heparin, black) and Compound2 (PC, red) in phase space along the first two principal eigenvectors.

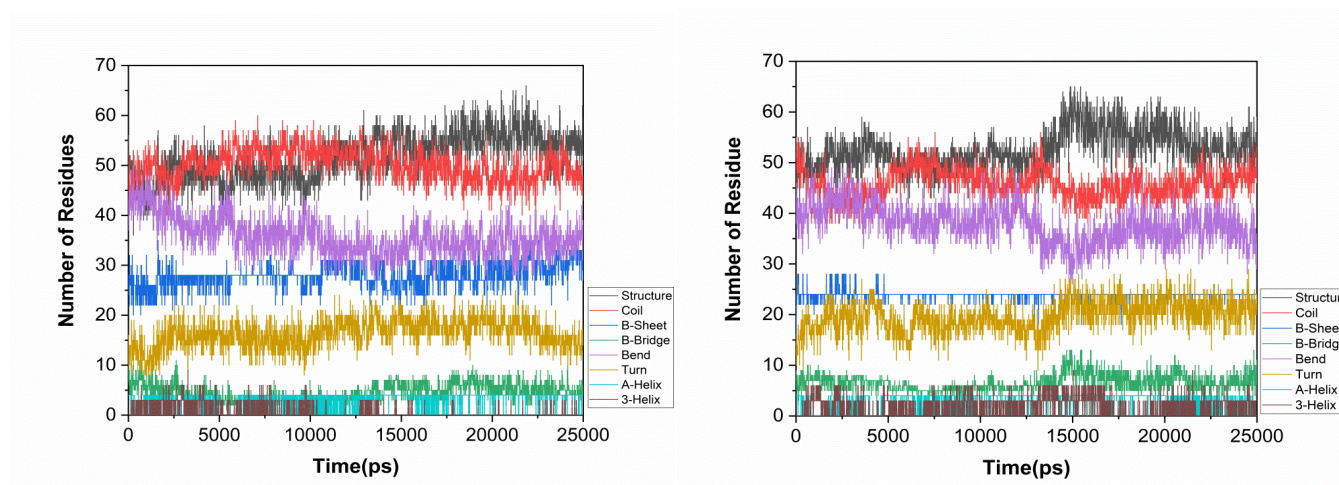


Figure 4.18: Secondary structure changes during the course of 25-ns MD simulation in (A) Heparin; (B) PC

4.11 Binding Free Energy Calculation

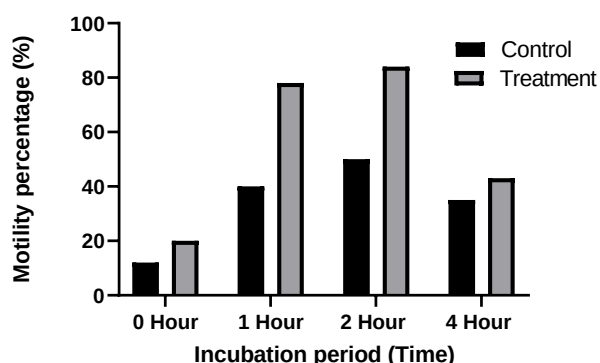
The binding free energy of protein-ligand complexes were calculated using *g_mmpbsa* tool based on molecular mechanics-based Poisson–Boltzmann Surface Area. This method was applied for various studies like stability of the protein, protein-ligand interaction analysis and protein design. The different type of interaction such as hydrophobic, hydrogen, electrostatics and pi-pi were formed rBSP1- heparin/PC complexes. In this study, 250 snapshots were taken from 25ns MD trajectories and estimated the binding energy for both complexes. The binding energy of rBSP1-heparin and rBSP1-PC complexes is -126.937 ± 95.414 KJ/mol, -45.485 ± 29.332 KJ/mol. The vdW energy was calculated that higher negative value of rBSP1-heparin complex as compare to rBSP1-PC complex. It was observed that rBSP1-heparin complex possesses higher negative value of vdW energy because of solvent accessible surface area energy (ΔG_{sasa}) and highest free energy due to Vander Waal and electrostatic interaction signifying the considerable hydrophobic interaction and stability of the rBSP1-heparin complex compared to rBSP1-PC complex (Table 4.4).

Table. 4.4: Binding free energies of Heparin and PC calculated using the g_mmpbsa method.

Energy components (kJ/mol)	Name of Ligand	
	Heparin	PC
Van Der Waal energy (ΔG_{vdW})	-151.065 ± 103.024	-82.019 ± 22.942
Electrostatic energy (ΔG_{ele})	-51.488 ± 44.724	-8.879 ± 16.056
Polar solvation energy (ΔG_{ps})	90.919 ± 67.678	55.579 ± 50.188
SASA energy (ΔG_{sasa})	-15.305 ± 10.014	-10.166 ± 2.529
Binding energy ($\Delta G_{binding}$)	-126.937 ± 95.414	-45.485 ± 29.332

4.12 Effect of recombinant buffalo BSP on *in-vitro* capacitation

The motility and acrosome status of spermatozoa were predicted by CASA and Hoechst staining and it was observed that there was significant ($P < .05$) increased in motility % of spermatozoa cultured in IVF media supplemented with @50 μ g/ml of rec-BSP1 compared to control group and other concentrations at 1 & 2 h sperm incubation. (Fig 4.19). Similarly, AR % in spermatozoa treated with 50 μ g/ml of recombinant BSP protein was increased significantly from 2 & 4 hours of incubation compared to control & other concentrations of protein treated groups (Fig.4.20).

**Figure 4.19:** Effect of 50 μ g/ml rBSP1 and heparin in different incubation period (hours) on motility percentage of buffalos spermatozoa

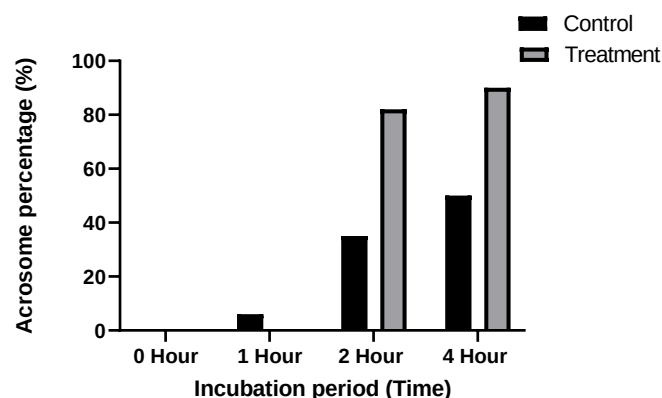


Figure 4.20: Effect of 50µg/ml rBSP1 and heparin in different incubation period (hours) on acrosome reaction percentage of buffalos spermatozoa

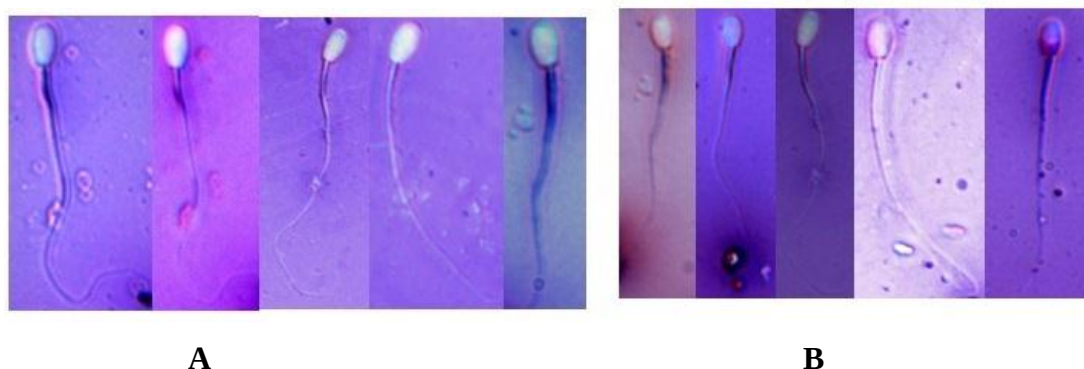


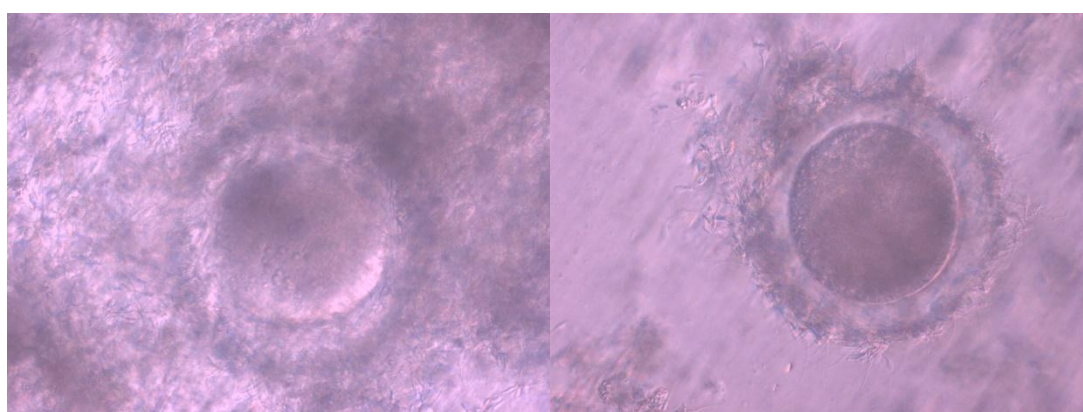
Figure 4.21: (A) Eosin-Nigrosine Staining of buffalo spermatozoa. All the live sperms head was clearly visible and showing bright heads in 50 µg BSP recombinant protein treatment group. (B) Eosin-Nigrosine staining of buffalo spermatozoa. All the head of sperms was clearly visible and showing dull heads in control (heparin) group.



Figure4.22: Trypan blue staining of buffalo spermatozoa. All the live sperms were clearly visible and showing bright heads in 50 µg BSP recombinant protein treatment group.

4.13 Effect of recombinant buffalo BSP on *in-vitro* fertilization

The effect of rec BSP on *in-vitro* embryo production was done through assessment of cleavage, blastocysts rate and number of hatched blastocyst in buffaloes, for this randomly allocated matured oocytes before presumptive zygotes formation as test using 20, 50, 100µg/ml and control group. Among three concentrations, in rec-BSP1 (50µg/ml) treated condition, the cleavage and blastocysts rates were significantly higher than the control. Similarly, number of hatched blastocysts were higher at concentration of 50µg/ml of rec-BSP1 (Fig.4.21 & Table 4.5). The few selected blastocysts derived from control and treatment group were used for Hoechst 33342 staining (Fig.4.22). It was observed that there was increase in cell number in blastocyst treated with 50µg/ml of rec-BSP1 compared to the control.



A

B

Figure 4.23: Co-incubation of capacitated spermatozoa and oocyte A. IVF (BO) medium supplemented with 50 µg/ml of rec-BSP1, B. IVF medium supplemented with 10 µg/ml heparin (control).

Table. 4.5: Effect of rec-BSP1 on *in vitro* buffalo embryo production

Concentration(µg/ml)	No. of oocytes	Cleavage rate (%)	Morula (%)	Blastocyst rate (%)	Hatched Blastocyst No.
Control/heparin	150	87 (58)	49 (56.32)	14 (16.09)	8 (9.19)
20	138	75 (54.34)	30 (40)	10 (13.33)	5 (6.66)
50	131	85(64.88)	55 (64.70)	22 (25.88)	17 (20)
100	138	64(46.37)	19 (29.68)	4 (6.25)	2 (3.12)
C.D.	8.33	13.93 NS*	9.73NS*	5.87S*	2.72S*

NS* Non significant S* Significant



Figure 4.24: A few representative images of hatched blastocyst in IVF a. Hatched/ expanded/ early blastocyst in control, b. Hatched/ expanded/ early blastocyst in IVF (BO) medium supplemented with 10µg/ml of rBSP1, c. Hatched blastocyst in IVF (BO) medium supplemented with 50µg/ml of rBSP1.

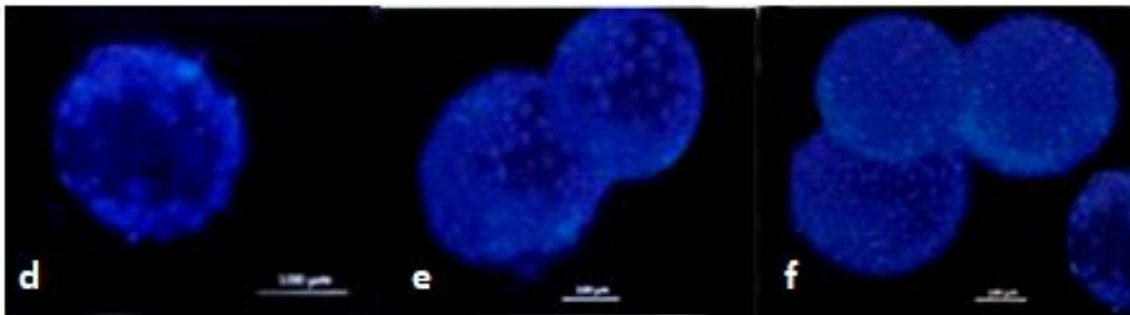


Figure 4.25: A few representative images of Hoechst staining of hatched blastocyst. d. H. staining of blastocyst in control, e. Hoechst staining of blastocyst IVF (BO) medium supplemented with 10µg/ml of rBSP1, f. Hoechst staining of blastocyst IVF (BO) medium supplemented with 50µg/ml of rBSP1

CHAPTER –5

Discussion

DISCUSSION

Artificial insemination (AI) and cryopreservation are important to enhance fertility in livestock. The seminal plasma contains many proteins which influence the level of fertility in bull. Mc Cauley et al., (1999) hypothesized that fertility of sub-fertile spermatozoa could improve by treating with seminal plasma taken from a fertile bull. Binder of Sperm Proteins (BSPs) is major proteins in seminal plasma of a bull that contributes 70% of total seminal plasma proteins. The BSP has a unique structure and present in multimeric isoforms and diverse functions such as capacitation, fertilization and protective action against stressful conditions.

Bovine seminal plasma (BSP) protein named as Binder of Sperm (BSP) Protein (Manjunath et al, 2009) are major protein represented 40-57% of total seminal plasma proteins. Among them BSP-A1/A2 (BSP1), BSP-A3 (BSP3), BSP30 (BSP5) contribute 47%, 3-5% and 3- 7% respectively. The BSP is composed of a unique N-terminal domain followed by two fibronectins type II (FN2) domains which are separated by a short polypeptide linker. The fibronectin domains have binding sites for different chemicals like heparin, phosphoryl choline, gelatin which are conserved in BSP family and functionally significant (Gwathmey et al., 2006, Murrey and Smith, 1997). The molecular weight & isoelectric points (pI) of BSP1/3 has 15-16.5kDa and 4.7-5.2, whereas molecular weight & isoelectric points (pI) of BSP5 has 28-30kDa and 3.9-4.6. (Manjunath et al, 1987; Densoyer et al, 1994). Among the member of BSP family BSP1 and BSP5 (most glycosylated) both contain neutral sugars, galactosamine, and sialic acid, whereas BSP3 contains galactosamine, less sialic acid but does not contain neutral sugar (Manjunath et al, 1987). The BSP forms a reservoir in oviductal epithelium where sperm store and interact to prolong the maintenance of survivability, motility of spermatozoa and prepare for capacitation (Gwathmey et al, 2006) and function as molecular chaperone in *in-vitro* for folding properly & stimulate sperm capacitation pathway (Sankhala and Swamy, 2010).

The BSP protein has important role in reproduction and it is enhanced the fertility in male animals. It is believed that it plays a significant role in selection of high quality sperm which leads to successful fertilization. However, there are no available reports on the use of buffalo recombinant BSPs *in-vitro* fertilization. Keeping in view of the lower

Discussion

rate of fertility and scarcity of the native proteins as native purification is difficult because of transient secretion. Therefore, the present investigation was proposed to produce recombinant BSP protein and showed its effect on *in-vitro* capacitation and fertilization.

The primers were designed for full length BSP gene using *Bubalus bubalis* binder of sperm 1 (BSP1) mRNA, complete CDS (Accession no. KR703587). Total RNA was extracted from seminal vesicles and synthesized cDNA using random hexamer primer. PCR amplification of target sequence was carried out using the cDNA as template and designed primers set. The amplicon with size of 468bp was purified from gel and cloned in pEASY blunt cloning vector. The positive recombinant clones were screened by visualizing its blue-white colour from LB/ampicillin/ X-gal/ IPTG plates & colony PCR. The nucleotide sequence analysis of BSP gene revealed that it was similar to the PDC109 sequence with identity of 99%. The sequence analysis revealed that N-terminal of protein is highly conserved while C-terminal was divergent. We identified the relatively predominant isoform of BSP and classified as BSP isoform1. The selected positive clones undergo RE digestion, ligation and subsequently cloned in expression vector (pET28a) and transformed in *E. coli* cell (BL21DE3) for production of rec-buBSP1. The induction concentration of 1 mM IPTG and incubation period of 22 hours at 37°C & 16°C were standardized for maximum production of the recombinant protein. The recombinant protein was 6x His tag fused to N-terminal of protein hence the recombinant protein was purified by Ni-NTA agarose column and produced the protein in soluble and active protein as a (6) thioredoxin tagged protein in *E. coli*. BL21 (DE3) cell and visualized in SDS PAGE with size of ~17 kDa. The recombinant protein was obtained maximum in soluble form approximately 2 mg protein as confirmed by SDS PAGE, Western blot analysis.

The semen samples with concentration @ 2-4million spermatozoa/ml in capacitation medium (BO) supplemented with different concentration of recombinant BSP proteins (20, 50 & 100 µg/ml) and semen without treatment with protein was used as a control to assess progressive motility, hyperactivity and acrosome reaction. The progressive motility, hyperactivity of spermatozoa assessed by swim up technique & CASA study and H& E, eosin & nigrosin, trypan blue staining were done for morphology of spermatozoa. Similarly, Hoechst staining was also performed to assess the acrosome status of spermatozoa. It was observed that the sperm motility was ranged

from 78 % to 84 % and a significant ($P < .05$) increase in IM% was noticed at concentration of @ 50 µg/ml of recombinant BSP proteins at 1 h of incubation. There was significant increase in AR % ($P < .05$) in sperm samples treated with @ 50 µg/ml of recombinant buBSP1 proteins at 2 & 4 hours of incubation compared to control & other concentrations of the recombinant protein treated groups.

Similarly, *In-vitro* Fertilization study was carried out in which the mature oocytes were co-incubated with processed frozen thawed capacitated semen in a Petri dish under controlled condition for union of male and female gametes, result in formation of pronuclei and zygote. The pellet containing spermatozoa was suspended in 1ml of the capacitation and fertilization BO medium supplemented with 20, 50, 100µg/ml recombinant BSP, 10 µg/ml heparin (control) and co-incubated with mature oocytes to assess the cleavage & blastocyst rate.

It was found that at concentration 50µg/ml of rec-buBSP, the cleavage, blastocysts rate significantly higher than control. Similarly, number of hatched blastocyst higher at concentration of @ 50µg/ml of rec-buBSP1. Then selected the blastocyst derived from control and *In-vitro* fertilization medium supplemented with @50µg/ml of rec-buBSP1 for Hoechst 33342 staining. The counting the total cell numbers day 9 blastocysts cells was done after Hoechst 33342 staining of 20-25 IVF blastocysts nuclei @ 10 ng/ml for 10 minutes and washed three times in DPBS. The stained embryos were mounted individually on microscopic slides with a cover slip in glycerol and examined the mounted preparation under UV. It was observed that there was increase of cell numbers in blastocyst cultured in IVF media supplemented with @ 50µg/ml of rec-buBSP1 compared to control. The cleavage of fertilized oocytes and their further development into blastocysts is an indication of the efficiency of an *in-vitro* embryo production. Recent studies reported that a concentration of 40µg/ml native BSP protein enhance early *in-vitro* embryo development (Rodriguez et al.2016). Accordingly, we planned to at least three doses (20, 50, 100µg/ml) of recombinant buBSP1 used on blastocyst development rate among them the concentration of 50µg/ml recombinant buBSP1 supplemented in the *in-vitro* culture medium and it was found that the concentration of the recombinant protein enhanced the blastocyst formation rate and number of hatched blastocysts along with increased total number of cells within blastocysts.

Conclusions

The rec-buBSP1 was produced in soluble fraction in its native form. Molecular docking experiment proved that buBSP-1 interacts with heparin with stronger affinity than PC. We further examined the role of buBSP-1 on capacitation of sperm in comparison to heparin. It was observed that buBSP1 significantly improved the capacitation. The rec-buBSP1 at a concentration of 50ug/ml exerted better effect on the rate of blastocyst formation in comparison to heparin and other treatments.

In conclusion, this study of the role of rec-BSP1 in reproductive function may recognize a new fertility factor that can be beneficial to enhance male fertility. Applicably, the research work suggests that the proposed homology model of heparin may be a suitable, consistent and robustness model with highest docking score seems to improve the activity of rec-BSP1 and ultimately use as a potent fertility factor which enhance *in vitro* embryo production.

CHAPTER –6

Summary and Conclusions

SUMMARY AND CONCLUSION

The reproductive success of a female animal depends upon fertility of a sire. Artificial insemination and cryopreservation are very important for using the sire genetic potential to enhance fertility. Ejaculated sperm possess seminal plasma which consists of factors that influence the level of fertility in a bull. The seminal plasma proteins have positive effect on fertility (Bellin et al., 1998; Henault and Killian, 1996). Mc Cauley et al., (1999) hypothesized that fertility of sub-fertile spermatozoa could improve by treating with seminal plasma taken from a fertile sample.

Binder of Sperm Proteins (BSPs) is one of the major proteins in seminal plasma in male which contribute around 70% of total seminal plasma proteins. BSPs have a unique structure which consists of 2 fibronectin domains separated by short chain peptide linker that conserve among BSP family. These 2 domains are characterized by binding to different biomolecules like heparin, HDL, glycosaminoglycan etc. These proteins present in multimeric isoforms and have diversified functions such as capacitation, fertilization and protective action against stressful conditions. BSPs are generally bound to epithelium of lower oviduct forming a sperm reservoir which influences viability, motility and fertility of spermatozoa. The reservoir also serves to reduce the incidence of polyspermy during fertilization and it is believed that it plays a significant role in selection of high quality sperm which leads to successful fertilization. However, there are no available reports on the use of buffalo recombinant BSPs in vitro fertilization. It is also a tedious process to isolate a native form of BSPs from live animal in a desire amount. Therefore, it is urgently necessary to produce a recombinant BSP in a heterologous system for evaluating the effectiveness to see the improving performances in in vitro condition such as its effect on increasing capacitation process and production of embryos. Keeping in view of the lower rate of fertility and scarcity of the native proteins, the present investigation was proposed to produce recombinant BSP protein and showed its effect on In Vitro capacitation & In Vitro Fertilization.

The primer pairs were designed for full length BSP gene using Bubalus bubalis binder of sperm 1 (BSP1) mRNA, complete CDS (Accession no. KR703587). Total RNA was extracted from seminal vesicles and cDNA was synthesized using random hexamer primer. PCR amplification of target sequence was carried out using the cDNA

Summary and Conclusions

as template and designed primers set. The amplicon with size of 468bp was purified from gel and cloned in pEASY blunt cloning vector. The positive recombinant clones were first screened by visualizing its blue-white colour from LB/ampicillin/ X-gal/ IPTG plates & colony PCR and further confirmed by RE digestion and sequencing. The nucleotide sequence analysis of BSP gene revealed that it was similar to the BSP sequence with an identity of 99%. The sequence analysis revealed that N-terminal of protein is highly conserved while C-terminal was divergent. The selected positive clones subsequently cloned in Expression vector (pET28a) and transformed in E. coli cell and induced with 1mM IPTG for production of recombinant protein. The induction concentration of 1 mM IPTG and incubation period of 20-22 hours at 16⁰C were standardized for maximum production of recombinant protein.

The recombinant proteins were 6x His tag fused to N-terminal of proteins hence the recombinant protein was purified by Ni-NTA agarose column & produced the protein in soluble & active protein as a his (6) thioredoxin tagged protein in BL21 (DE3) cell & checked in SDS PAGE with size of ~17 KD. The recombinant protein was obtained maximum in soluble form @ around 2 mg protein as confirmed by SDS PAGE, Western blot analysis. The assessment of semen quality (motility and acrosome reaction) was done by treating the spermatozoa with different concentration of recombinant buBSP1 protein (20, 50 & 100µg/ml). Similarly, three doses (20, 50 & 100 µg/ml) of recombinant buBSP1 was used on In Vitro Fertilization study. It was found that there is significant increase in individual motility percentage, acrosome reaction (AR %) @ 50µg/ml rec-buBSP1 protein treated for 1-4 hours of incubation and the best concentration of rec-buBSP1 protein used for In Vitro Fertilization (IVF). The concentration of 50µg/ml of rec-buBSP1 supplemented in In Vitro Culture medium enhanced the blastocyst formation rate along with increased the total number of cells within resulting blastocysts & increased the number of hatched blastocysts.

Conclusion

The research work carried out under entitled of the research problem i.e. “Expression and Characterization of re-buBSP1 and its effect on *In Vitro Fertilization*” is highly applicable in the area of male fertility to enhance reproduction efficiency. The detail mechanism by which the appropriate concentration of the recombinant protein is promoting the blastocyst rate is further researchable.

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Annexures

A1. General Reagents and Salt Solutions**1. Phosphate buffer saline (pH 7.2)**

Sodium chloride (NaCl)	8.0 g
Potassium chloride (KCl)	0.2 g
Sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)	1.44 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Distilled water upto	1000 ml
pH is adjusted to 7.2 with 1N HCl/NaOH	

1. 30 % acrylamide stock solution

Acrylamide	30 g
N'-N'-bis-methylene acrylamide	0.82 g
Distilled water up to	100 ml
Filter the solution through Whatman No 1 filter paper and stores at 4°C in a dark bottle.	

2. Lower gel buffer

SDS	0.4 g
Tris buffer	18.17 g
Distilled water up to	100 ml
pH is adjusted with 1N NaOH/HCl to 8.8 and stores at 4°C	

3. Upper gel buffer

Sodium dodecyl sulphate (SDS)	0.4 g
Tris buffer	6.0 g
Distilled water up to	100 ml
pH is adjusted with 1N NaOH/HCl to 6.8 and stored at 4°C	

4. 10% ammonium per sulphate

Ammonium per sulphate	100 mg
Distilled water	1 ml

The solution was discarded after 7 days and prepared freshly.

5. SDS running buffer

SDS	1 g
Glycine	14.26 g
Tris buffer	3.03 g
Distilled water up to	1000 ml

6. 2 x Sample buffer

Glycerol	20 ml
SDS	4.6 g
Tris buffer	1.52 g
DDT	1.56 g
Bromophenol blue	0.015 g
Distilled water up to	100 ml

Tris is dissolved in 30 ml of distilled water followed by the addition of SDS, glycerol and bromo phenol blue.

pH is adjusted with 1N NaOH/HCl to 6.8 and stores at 4°C

7. Staining solution

Coomassie brilliant blue R-250	100 mg
Methanol	45.4 ml
Acetic acid	9.0 ml
Distilled water upto	100 ml

First the dye was dissolved in methanol and then acetic acid and water were added. The entire mixture was stirred. Finally the staining solution was passed through Whatman No 1 filter paper.

8. Destaining solution-7.5% Glacial acetic acid

Glacial acetic acid	7.5 ml
Distilled water upto	100 ml

9. Preparation of gels

a)	Separating gel (10%)	10 ml
	30% acrylamide	3.33 ml
	Distilled water	4.16 ml
	Lower gel buffer	2.5 ml
	TEMED	10 μ l
	10% APS	30 μ l
b)	Stacking gel (5%)	5ml
	30% acrylamide	0.79 ml
	Distilled water	2.96 ml
	Upper gel buffer	1.25 ml
	TEMED	5 μ l
	10% APS	1.5 μ l

1. 0.5 M Ethylene diamine tetra acetate (EDTA), pH 8.0

EDTA disodium salt	18.61 g
Distilled water	80 ml

NaOH pellets added while stirring the solution to adjust pH 8.0. Final volume made to 100 ml, sterilized by autoclaving and store at 4⁰C

2. Tris-Acetate-EDTA (TAE) stock solution (50X)

Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA, pH 8.0	100ml
Distilled water	1000ml

Sterilized by autoclaving and stored at RT. For working solution (1X), stock TAE

was diluted in water.

3. Ethidium bromide solution (10mg/ml)

Ethidium bromide	10mg
Distilled water	1ml

1. Luria-Bertani (LB) broth

LB broth powder (Himedia, Mumbai) (Casein hydrolysate 10g, Yeast extract 5g, NaCl 10g)	25g
Distilled water	1000ml

Autoclaved and stored at 37°C.

2. LB agar

LB agar powder (HiMedia) (Casein hydrolysate 10g, Yeast extract 5g, NaCl 10g, Agar 15g)	40g
Distilled water	1000ml

Heated to melt agar in the water and then autoclaved.

3. 1M Isopropyl thio-β-D-galactoside (IPTG)

IPTG	0.238g
Distilled water	1 ml

Filter sterilized and stored at -20°C. For working 1M IPTG was diluted 10 times.

4. Ampicillin

Ampicillin sodium salt	100 mg
Distilled water	1ml

Filter sterilized and stored at 4°C.

5. X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) 20 mg/ml stock

X-gal 50 mg

DMSO (dimethyl sulfoxide) 2.5ml

Wrapped in foil and stored at -20°C.

1. Alkaline lysis solution I

Glucose 0.9 g

Tris-Cl 0.303 g

EDTA 0.372 g

Distilled water up to 100 ml

pH was adjusted to 8.0 using 1N NaOH/ HCl. Autoclaved the solution and store at 4°C.

2. Alkaline lysis solution II

Sodium hydroxide (NaOH) 0.8 g

SDS 1.0 g

Distilled water up to 100 ml

Autoclaved and store at room temperature.

3. Potassium acetate solution (5M)

Potassium acetate 29.44g

Distilled water up to 60 ml

4. Alkaline lysis solution III

Potassium acetate solution (5M) 60 ml

Glacial acetic acid 11.5 ml

Distilled water 28.5 ml

Autoclaved and store at 4°C

Annexure

1. Lysis buffer

Sodium dihydrogen phosphate (NaH_2PO_4)	1.2 g
Tris-Cl	0.12 g
Urea	48.05g
Distilled water up to	100 ml
pH was adjusted to 8.0 using 1N NaOH/ HCl.	

2. Wash buffer

Sodium dihydrogen phosphate (NaH_2PO_4)	1.2 g
Tris-Cl	0.12 g
Urea	48.05g
Distilled water up to	100 ml
pH was adjusted to 6.3 using 1N NaOH/ HCl.	

3. Elution buffer

Sodium dihydrogen phosphate (NaH_2PO_4)	1.2 g
Tris-Cl	0.12 g
Urea	48.05g
Distilled water up to	100 ml
pH was adjusted to 4.5 using 1N NaOH/ HCl.	

4. OOCYTE COLLECTION AND MATURATION MEDIA

Normal Saline

Sodium chloride	9 g
Penicillin G	0.06g
Distilled water	1000ml

Aspiration Medium (for 200-250 ovaries)

TCM -199 (Hepes modified)	50ml
BSA	0.15g

L-glutamine	0.005g
Gentamycin sulphate	50µg/ml

Washing medium

TCM -199 (Hepes modified)	36ml
FBS @10%	4ml
Sodium pyruvate	0.0036g
L-glutamine	0.004g
Gentamycin sulphate	50µg/ml

Maturation medium

Washing medium	9.5ml
Porcine FSH	5µg/ml
Follicular fluid	0.5ml
Estradiol 17-β	5µg/ml

5. IN VITRO FERTILIZATION MEDIA**Brackett & Oliphant (BO) medium****Solution-A (Stock)**

Sodium chloride	4.3092 g
Potassium chloride	0.1974g
Calcium chloride dihydrate	0.2171g
Magnesium chloride hexahydrate	0.0697g
Sodium hydrogen phosphate dehydrate	0.0840g

Dissolved the above component in 500ml distilled water. Mix 0.05ml of 0.5% phenol red for coloring and indication pH of the solution. Add penicillin (50 IU/ml) and streptomycin (5µg/ml) for preservation for longer period.

Solution-B (Stock)

Sodium bicarbonate	2.5873 g
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Dissolved it in 200ml distilled water. Mix 0.01ml of 0.5% phenol red for coloring and indication pH of the solution. Add penicillin (50 IU/ml) and streptomycin (5µg/ml) for preservation for longer period.

Working BO medium (50ml)

Solution-A	38ml
Solution-B	12ml
Heparin	10 µg/ml
Sodium pyruvate	0.0068g
Caffeine sodium benzoate	0.0971g

(Heparin can be used 50-100 µg/ml solution)

Working BO medium for capacitation & fertilization (10ml)

Working BO medium	10ml
BSA (Fatty Acid free)	0.1g

6. IN VITRO EMBRYO CULTURE MEDIA**Washing medium for presumed Zygotes**

mCR2aa	9.0ml
FBS	1.0ml
Gentamycin sulphate	50 µg/ml

In vitro culture Medium

mCR2aa	20.0ml
BSA (Fraction V) @ 0.6%	0.12g
Gentamycin sulphate	50 µg/ml

Composition of Modified Charles Rosenkrans-2 Medium with Amino Acids (100ml aliquots)

NaCl	0.6329 g
NaHCO ₃	0.2091g
NEAA	1ml
EAA	2ml
Glutamine	0.0146g
KCL	0.0216g
Hemi calcium lactate	0.0272g
Sodium pyruvate	0.0055g
Glycine	0.0037g
Alanine	0.0044g
Glucose	0.0180g
Gentamycin	mCR2aa 9.0ml
FBS	1.0ml
Gentamycin sulphate	50 µg/ml
BSA(Fraction-V)	@0.6%
Phenol red	Mix 0.01ml of @0.6%
Distilled water (make final volume)	100ml