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ARTICLE

Expression of recombinant buck (*Capra hircus*) spermadhesinin a prokaryotic system

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ABSTRACT: (Expression of recombinant buck (*Capra hircus*) spermadhesin in a prokaryotic system). The low purification efficiency and the incomplete characterization of buck spermadhesins (Bdhs) prompted us to establish an effective system to produce recombinant Bdhs (rBdhs). The Bdh-4 cDNA was inserted in a prokaryotic expression plasmid pTrcHis TOPO to produce a His_6 fusion protein in *E. coli* Top10 cells. The recombinant clones were selected by growth in ampicillin-containing medium, PCR amplifications and nucleotide sequencing. The recombinant protein synthesis was monitored by SDS-PAGE followed by immunoblotting using a monoclonal anti-His antibody. The expression of the rBdh-4 was achieved at 0.1 to 2.0 mM IPTG after 2 to 6 h of induction. A greater production of rBdh-4 ($P < 0.001$) was obtained with 0.1 mM IPTG after 2 h of induction. The apparent molecular weight of rBdh-4 was 15.85 ± 0.09 kDa. This result agrees with the theoretical molecular weight of 16.5 kDa predicted from the nucleotide sequence. In conclusion, an effective rBdh-4 expression system was established in order to provide a good tool for studying the biofunctions of buck spermadhesins.
Key words: semen, recombinant protein, cDNA.

RESUMO: (Expressão da espermadesina recombinante de bode (*Capra hircus*) em sistema procariótico). A baixa eficiência de purificação e a incompleta caracterização das espermadesinas de bode (Bdhs) nos levou a estabelecer um sistema efetivo para produzir as Bdhs recombinantes (rBdhs). O cDNA da Bdh-4 foi inserido no plasmídeo de expressão procariótico pTrcHis TOPO para produzir uma proteína de fusão His_6 em células de *E.coli* Top10. Os clones recombinantes foram crescidos em meio contendo ampicilina, amplificação por PCR e sequenciamento de nucleotídeos. A síntese da proteína recombinante foi monitorada por SDS-PAGE seguida por imunobloting usando anticorpo monoclonal anti-His. A expressão da rBdh-4 foi conseguida de 0,1 a 2,0 mM de IPTG e depois de 2 a 6 H de indução. A maior produção da rBdh-4 ($P<0,001$) foi obtida com 0,1 mM de IPTG depois de 2 h de indução. O peso molecular aparente da rBdh-4 foi 15,85 ± 0.09 kDa. Este resultado está de acordo com o peso molecular teórico de 16,5 KDa predito pela sequencia de nucleotídeos. Em conclusão, um sistema efetivo de expressão da rBdh-4 foi estabelecido para fornecer uma boa ferramenta para estudos biofuncionais das espermadesinas de bode.
Palavras-chave: sêmen, proteína recombinante, cDNA.

INTRODUCTION

Seminal plasma, the fluid in which mammalian spermatozoa are suspended in semen, is a complex mixture of secretions originating from the epididymis and accessory glands, Fournier-Delpech & Thibault (1993). The protein composition of seminal plasma varies from species to species. These components have important effects on sperm function, influencing the fertilizing ability of spermatozoa, and exert effects on female reproductive physiology, Töpfer-Petersen *et al.* (2005). An important group of seminal plasma proteins belongs to a group of lectin-like proteins called spermadhesins. Spermadhesins are a group of polypeptides of 12– 16 kDa found in seminal plasma and peripherally associated with the sperm surface of ungulates, such as pigs, bulls, rams, stallions and bucks (Bergeron *et al.* 2005, Calvete *et al.* 1995, Reinert *et al.* 1996, Tedeschi *et al.* 2000, Teixeira *et al.* 2002, Teixeira *et al.* 2006), which are able to interact with some sugar containing receptors on cell surfaces. The capacity to bind to sugar moieties is a biological activity characteristic of lectins and lectin-like proteins. All animal lectins contain a carbohydrate-recognition domain (CRD) in their amino acid sequence. However, spermadhesins differ structurally from the majority of lectins. They show a distinct protein domain called CUB, a widespread 110- amino acid module, which was named after the structural analysis of three proteins in which the CUB domain was first identified (complement subcomponents-C1r/C1s, embryonic sea urchin protein-Uegf, and bone morphogenetic protein 1-Bmp1). This domain consists of a sandwich made up of two sheets, each containing four anti-parallel strands and one parallel strand (Varela *et al.* 1997).

The low purification efficiency and the incomplete

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characterization of Bdhs (Cajazeiras *et al.* 2009, Mello *et al.* 2009), as well as the potential multiplicity of biotechnological applications of proteins from the spermadhesin family, prompted us to establish an effective expression system to produce recombinant Bdhs. *Escherichia coli* is the preferred host for recombinant protein expression for various studies because it is rather easy to genetically manipulate, it is relatively inexpensive to culture, and expression is fast, typically producing protein in a single day. Thus, in the present study, an effective prokaryotic recombinant Bd expression system was established in order to provide a solid basis for future functional studies and biotechnological applications of rBdhs.

MATERIAL AND METHODS

Animal care

The animal used for this study was anesthetized and sacrificed according to the guidelines of animal care (Langley 1997).

Cloning of Bdh-4 cDNA

cDNA coding for spermadhesin Bdh-4 was produced according to the method described by Mello *et al.* (2008). Briefly, total RNA was isolated from a single sexually mature buck of undefined breed using the Trizol reagent (Invitrogen, USA), following the manufacturer's instructions. Poly(A^+)-RNA was obtained from the total RNA by affinity chromatography on oligo(dT) cellulose, using an mRNA purification kit (Invitrogen, USA). First-strand cDNA was synthesized by reverse transcription coupled to PCR using a 3′-adaptor-Oligo(dT)18 (Clontech, USA) and Moloney murine leukemia virus reverse transcriptase (M-MLV RT) purchased from Promega (Madison, USA). Following cDNA synthesis, the 3′-rapid amplification of cDNA end (3′-RACE) was performed. Cloning of the spermadhesin gene was performed with the pGEM-T Easy Vector System and *E. coli* JM109 (Promega, USA). The identity of Bdh-4 cDNA was assessed by nucleotide sequencing performed on Mega BACE 750 DNA Analysis System (GE Healthcare, USA). The assembled cDNA sequence of Bdh-4 clones spanned 477 bp (GenBank accession number EF157971).

Construction of prokaryotic expression vector

The coding region of Bdh-4 was amplified by PCR using, as a template, the pGEM-T Easy cloning vector containing the cDNA obtained previously. Plasmid extraction and purification was carried out following the manufacturer's instructions of the GFX Micro Plasmid Prep Kit (GE Healthcare, USA). Purified plasmids were spectrophotometrically quantified. The forward primer included the first N-terminal residues and the reverse primer included the last C-terminal residues of the mature protein and two stop codons. The primers were: 5′-TGTGGGGGSGTCCACAGA-3′ (forward) and 5′-AAGATTGCTTACTCCTGA-3′ (reverse). PCR amplification was performed in 20-µL reaction volumes containing 1 U of Pfu DNA polymerase (Stratagene, USA), 50 ng of purified plasmid, 4 mM dNTPs and 4 µM of each primer in the PCR-buffer provided. The PCR amplification protocol included 30 cycles of denaturation (95°C for 50 s), annealing (55 °C for 50 s), and extension (72 °C for 50 s) followed by a final extension for 8 min at 72 °C. The products were separated on a 1 % agarose gel, stained with 0.5 mg/mL ethidium bromide, and viewed with a UV transilluminator. The amplified fragments were subcloned using a pTrcHis TOPO TA expression kit (Invitrogen, USA), according to the manufacturer's instructions. Briefly, 3 µL of PCR product was linked to 10 ng of pTrcHis TOPO in a final reaction volume of 5 µL. The recombinant plasmid produced was utilized for transformation of Top10 One Shot in a proportion of 1:25 (v: v). The cells were grown in a nonselective medium and plated on ampicillin-containing medium.

PCR colony

Positive clones, selected by growing the transformed cells in LB medium containing 50 µg/mL ampicillin, were confirmed by PCR using the insert-flanking pTrcHis Forward and pTrcHis Reverse primers (Invitrogen, USA). PCR was carried out using a Mastercycler ep Gradient S (Eppendorf, Germany), and amplicons were separated on a 1 % agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized with a UV transilluminator.

Recombinant spermadhesin expression analysis

One positive *E. coli* Top10 selected clone, shown by PCR amplification and nucleotide sequence to contain the correct Bdh-4 construct, was grown overnight at 37 °C in LB medium containing 50 µg/mL ampicillin, followed by a 1:50 (v/v) dilution in the same medium. Cells were grown to an optical density of 0.6 at 600 nm and were induced to express rBdh-4 by the addition of isopropyl-D-thiogalactoside, IPTG (USB Corporation, USA), to final concentrations ranging from 0.1 to 2 mM. For immunoblotting analysis of successful rBdh-4 production, cells from 1 mL aliquots of the cultures were harvested after 2, 4 and 6 h of IPTG induction by centrifugation at 6000 ×g for 10 min and the pellets were stored frozen at -20 °C.

SDS-polyacrylamide gel electrophoresis

Pellet samples were submitted to the SDS-polyacrylamide gel electrophoresis standard method (Laemmli, 1970) using 12.5 % polyacrylamide gel. Protein extracts were boiled in loading buffer (50 mM Tris-HCl, pH 6.8, 2 % SDS, 0.1 % Bromphenole Blue, 10 % Glycerin) for 5 min and 18 µl were loaded in each lane. High range protein standards (GE Healthcare, USA) were used as molecular weight markers.

Western blot analysis

SDS-PAGE-separated proteins were then transferred to polyvinylidene membranes (GE Healthcare, USA) at a constant voltage of 25 V at room temperature for 2 h using a miniVE Blot Module apparatus (GE Healthcare, USA) and 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20 % methanol (v/v) as a transfer buffer. Nonspecific binding sites were blocked by incubating the membranes with 5% nonfat dry milk (w/v) in TTBS (100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1 % Tween 20) overnight at 4 °C. Subsequently, the membranes were incubated with a 1:1.500 (v/v) dilution of the monoclonal anti-polyhistidine antibody clone HIS-1 (Sigma-Aldrich, St. Louis, MO, USA) for 150 min at room temperature. After washing three times with TTBS, the membranes were incubated for 60 min with a 1:10.000 (v/v) dilution of anti-mouse IgG-alkaline phosphatase (Sigma-Aldrich, USA), and then washed three times with TTBS and once with TBS (100 mM Tris-HCl, pH 7.5, 150 mM NaCl). The rBdh-4-His_6 was viewed by the addition of Sigma Fast NBT/BCIP buffered substrate (Sigma-Aldrich, USA) and the reaction was stopped with water.

Solubilization of inclusion bodies

In order to search for the putative insoluble form of rBdh-4-His_6, the cells obtained from 50 mL of culture were harvested by centrifugation at 6000 ×g for 10 min. The pellet was suspended in 8 mL of native buffer (50 mM sodium phosphate, 500 mM NaCl, pH 8.0) or in 8 mL denaturing buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8). Cells were lysed by sonication (5 cycles of 15 s) in an ice bath to reduce viscosity. The lysates were centrifuged at 6000 ×g for 30 min at 4 °C. The clear supernatant (soluble fraction) and the remaining pellet (insoluble fraction) containing inclusion bodies were collected and analyzed by the immunoblotting method as described above.

Image analysis and statistics

The image of each immunoblot membrane was recorded and the intensity of each band was assessed by densitometry using an image analysis program (ImageQuant TL 2005, Amersham Biosciences, USA). Quantitative analysis of the expression of rBdh-4 was performed by normalizing the data to the density of the band produced in the presence of 2 mM IPTG, after 2 h induction, which was assigned a value of 1.0. The quadratic curve model was utilized to calculate the apparent molecular weight of the protein. The data were expressed as means ± SEM of at least three replicates for each assay on different membranes. The program used for statistical analysis was GraphPad InStat 3.06 (GraphPad Software, USA). Data were compared using Tukey´s significant difference procedure and statistical significance was considered at $P < 0.001$.

RESULTS

In the present work, we subcloned the cDNA of Bdh-4. We used, as a PCR template, the clones produced previously (Mello *et al.*, 2008), which contain cDNA inserts of 477 bp. In particular, the Bdh-4 cloned fragments comprised a 342 bp ORF including the stop codon and a 278 bp 3´-untranslated region.

In order to subclone the Bdh-4 coding region corresponding to 113 amino acids into the pTrcHis TOPO vector, we used two primers designed to amplify only the ORF. The recombinant plasmid pTrcHis-Bdh-4 (Fig. 1A) was transformed into *E. coli* and 19 clones were selected by growth in ampicillin-containing medium. The clones containing the construct were identified by PCR, using insert-flanking primers and then confirmed by nucleotide sequencing. The length of all PCR products was approximately 350 bp (Fig. 1B). All the clones investigated contained DNA inserts compatible with the size of the ORF of the Bdh-4 spermadhesin

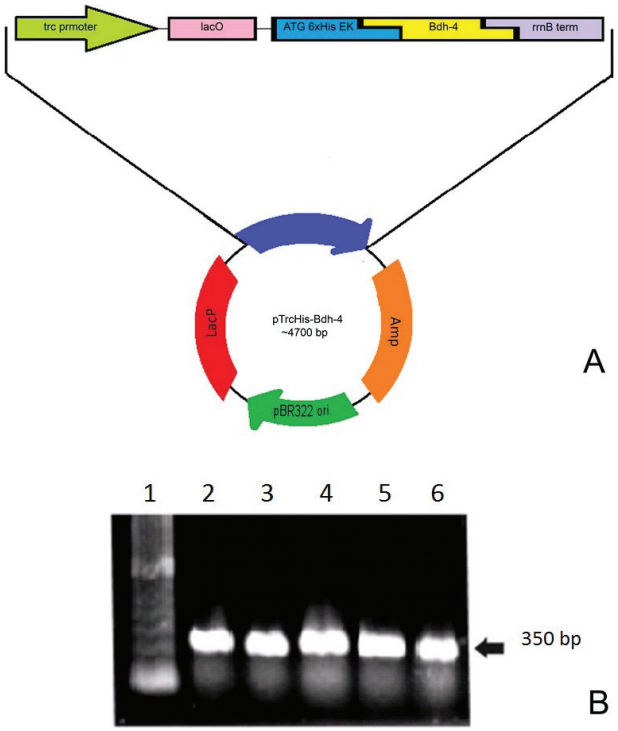

Figure 1. Construction and identification of pTrcHis-Bdh-4 prokaryotic expression vector. A. Schematic diagram of the features of pTrcHis-Bdh-4 vector. Abbreviations: pBR322 ori, origin of replication; Amp, ampicillin resistance gene; *LacI*^{q}, *lac* repressor gene; *lacO, lac* operator; ATG, start codon; 6×His, sequence coding for polyhistidine tag; EK, enterokinase cleavage site; Bdh-4, Bdh-4 cDNA fragment; *rrnB* term, transcription termination sequence. B. Identification of recombinant plasmid pTrcHis-Bdh-4 by polymerase chain reaction (PCR) analysis. *Lane 1*, High-Range Rainbow Molecular Weight Markers (GE Healthcare); *lanes 2* to *6*, PCR amplification products of pTrcHis-Bdh-4 using insert-flanking primers.

utilized in the present study.

Based on nucleotide sequencing of the expression vectors produced, one clone was selected for expression of the recombinant protein. The cDNA and the plasmid insert-flanking regions were completely identified (Fig. 2). The inserted fragment showed 100 % similarity with the corresponding region of Bdh-4 cDNA previously described (GenBank accession number EF157971). Additionally, the inserted cDNA was in frame with the start codon ATG of the pTrcHis-TOPO vector. This fact ensures a correct translation and the production of a true Bdh-4 amino acid sequence. The pTrcHis-Bdh-4 vector produced encodes a polypeptide chain of 134 amino acids, of which 113 residues correspond to rBdh-4.

The expression of the spermadhesin His_6 fusion protein was achieved at IPTG concentrations ranging from 0.1 to 2.0 mM and after 2 to 6 h of induction (Fig. 3). The recombinant protein synthesis was monitored by SDS-PAGE under reducing conditions followed by immunoblotting using monoclonal anti-His antibody.

His_6-Bdh-4 expression was induced at 37 ºC, and the apparent molecular weight was 15.85 ± 0.09 kDa (n=35), calculated by image analysis of the membranes. The relative protein production was also compared the by densitometric analysis of the intensity of each band on the membrane images (Fig. 4). A significantly greater production of rBdh-4 ($P < 0.001$) occurred in the presence of 0.1 mM IPTG after 2 h of induction. Among the induction times investigated, 6 h showed the lowest levels of rBdh-4 production, where no difference was seen between the various concentrations of IPTG tested ($P > 0.001$).

In order to examine the distribution of expressed rBdh-4 protein in soluble and insoluble fractions, both the supernatant and pellet of cell lysates after sonication in native buffer were analyzed (Fig. 5A). Samples were also analyzed by SDS–PAGE under reducing conditions and subsequent immunoblotting using a monoclonal anti-His antibody. A single band corresponding to His_6-Bdh-4 was produced after IPTG induction (Fig. 5A, lanes 1 and 2) and was almost insoluble in native

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gcnnacgtgtttgtatcttgtacgtgggcccgctgngncggntgctgaggctggtgccag  60

gctgcgaagatgaagctgtccagcgtcatcccttgggccttgctgctcagcacagccaca 120
          M  K  L  S  S  V  I  P  W  A  L  L  L  S  T  A  T
ctggtttcaacagaatcggatgaagacactagaaaatgtgggggcgtccacagagacttc 180
  L  V  S  T  E  S  D  E  D  T  R  K  C  G  G  V  H  R  D  F
tctgggaggatctccagcagtttctcatgggggccaaagtgtacctggaccatcctcttg 240
  S  G  R  I  S  S  S  F  S  W  G  P  K  C  T  W  T  I  L  L
aagagcggttatacagttgtactgacaattccatttctcagcctcaactgtaatgaagag 300
  K  S  G  Y  T  V  V  L  T  I  P  F  L  S  L  N  C  N  E  E
gatgtggaaatcatagacgggctgccagacagtactacatttgggaggttctgttcaggg 360
  D  V  E  I  I  D  G  L  P  D  S  T  T  F  G  R  F  C  S  G
ggacccctggtgtttaaatcttcttccaatgtcatgaccgtgaaatactacagaagttcc 420
  G  P  L  V  F  K  S  S  S  N  V  M  T  V  K  Y  Y  R  S  S
aaccagccagtatctccttttgatatattttactacgagcgtccaacagcttagtag     477
  N  Q  P  V  S  P  F  D  I  F  Y  Y  E  R  P  T  A stop stop
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Figure 2. Nucleotide sequence of pTrcHis-Bdh-4 expression vector and translated amino acid sequence. The plasmid vector is shown by underlined letters. The two consecutive stop codons are enclosed in gray letters. Nucleotide sequence encoding the N-terminal region of Bdh-4 is shown in bold letters.

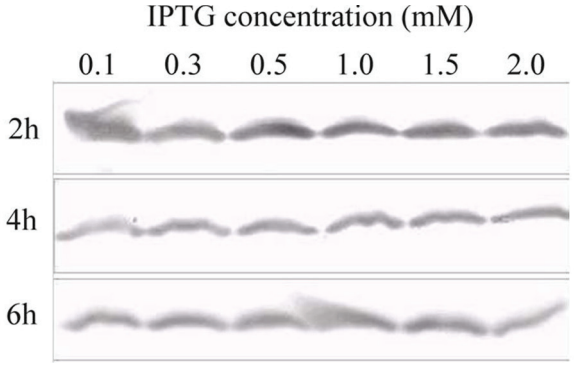

Figure 3. Expression analysis of His_6-fusion rBdh-4 spermadhesin in *E. coli*. Total proteins of pTrcHis-Bdh-4 transformant were submitted to 12.5% SDS-PAGE and analyzed by immunoblotting using monoclonal anti-His antibody. Cell samples were collected after 2, 4 or 6 h of IPTG induction at a final concentration ranging from 0.1

buffer (Fig. 5A, lanes 3 and 4). Thus, the synthesized rBdh-4 fusion protein was obtained largely in the insoluble fraction of the bacterial extract, as inclusion bodies. However, after cell sonication in denaturing buffer, approximately half of the total amount of rBdh-4 was dissolved (Fig. 5B, lanes 3 and 4).

DISCUSSION

A number of central elements are essential in the design of recombinant expression systems (Baneyx 1999). Recombinant expression plasmids require a strong transcriptional promoter to control high-level gene expression and a suitable repressor to minimize basal transcription in the absence of inducer. The most common inducer is the sugar molecule IPTG,(Hanning & Makrides 1998).

E. coli is the preferred host for heterologous protein

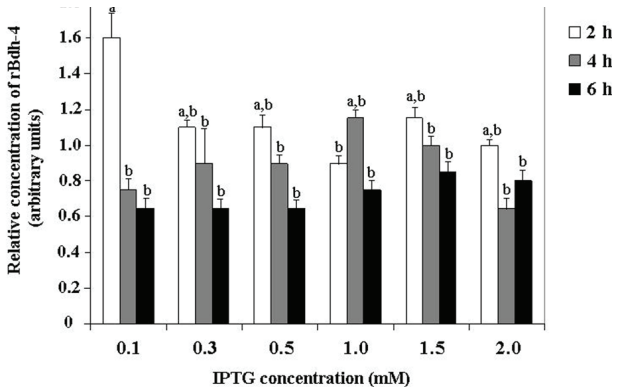

Figure 4. Effects of IPTG concentration and time of induction on relative abundance of His_6-fusion rBdh-4 spermadhesin in *E. Coli* expression system. Bacterial samples were collected after 2 h (white bars), 4 h (gray bars) or 6 h (black bars) of IPTG induction at a final concentration ranging from 0.1 to 2.0 mM. Bars with different superscripts differ significantly ($P < 0.001$).

Figure. 5. Extraction of His_6-fusion rBdh-4 produced in *E. coli* with native (A) or denaturing (B) buffer. Total proteins of pTrcHis-Bdh-4 transformant were submitted to 12.5% SDS-PAGE and analyzed by immunoblotting using monoclonal anti-His antibody. Samples of bacterial culture before (lane 1) and after (lane 2) IPTG induction, as well as the insoluble (lane 3) and soluble (lane 4) fractions were used.

production for several studies. However, some proteins simply fail to be expressed in *E. coli*, or are expressed but in an insoluble form as inclusion bodies. Inclusion bodies are a set of structurally complex aggregates often believed to occur as a stress response when recombinant protein is expressed at high rates (Van den Berg *et al.* 1999). The insoluble recombinant protein normally enriches the inclusion bodies by 50–95 % of the protein material (Carbonnel & Villaverde 2002). It is known that the formation of inclusion bodies in recombinant expression systems is the result of an imbalance between *in vivo* protein aggregation and solubilization (Jonasson *et al.* 2002). His_6-Bdh-4 expression was induced and the apparent molecular weight was 15.85 ± 0.09 kDa. This result agrees with the theoretical molecular weight. Additionally, the rBdh-4 molecular weight is in accordance with that described for all spermadhesin members, ranging from 12 to 16 kDa (Töpfer-Petersen *et al.* 1998).

The prokaryotic expression system constructed in the present work allowed for the efficient production of the spermadhesin Bdh-4, in the insoluble form, in *E. coli*. In addition, the maximum production of rBdh-4 was obtained after at 2 h of induction with IPTG. Thus, the longest induction period (4 to 6 h) likely leads to the degradation of the expressed protein. This is possible because inclusion bodies are not inert aggregates but act as a transient reservoir for loosely packaged folding intermediates *in vivo* (Carrio & Villaverde 2001). As shown in the present assay, the inclusion body aggregates can be solubilized using detergents such as urea. Subsequently, native protein can be prepared by *in vitro* refolding from solubilized inclusion bodies either by dilution, dialysis or on-column refolding methods, Sørensen *et al.* (2003).

Recently, recombinant boar AQN-1 and bull aSFP spemadhesins were successfully produced in a prokaryotic system by Ekhlasi-Hundrieser *et al.* (2008). These authors demonstrated that recombinant wild-type and some mutant spermadhesins exhibited similar mannose-binding characteristics, when compared to seminal plasma-isolated ones. Similarly, our expression system was constructed to produce an insoluble spermadhesin His_6 fusion protein in *E. coli* Top10 cells. Thus, we propose that, after solubilization, rBdh-4 keeps the same putative biological activity as Bdhs.

After affinity purification, the production of anti-rBdh-4 antibodies is more feasible. These antibodies will serve as a tool to access the expression level and the function of native Bdh-4 from buck seminal plasma. Additionally, the direct application of rBdhs has great potential with regard to enhancing our knowledge of buck reproductive mechanisms and further application in reproductive biotechnologies.

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