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Purified and Refolded Recombinant Bonnet Monkey (*Macaca radiata*) Zona Pellucida Glycoprotein-B Expressed in *Escherichia coli* Binds to Spermatozoa[1]

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ABSTRACT

Bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-B (bmZPB), excluding the *N*-terminal signal sequence and the *C*-terminus transmembrane-like domain, has been expressed in *Escherichia coli* as polyhistidine fusion protein. A requirement of 4 M urea to maintain the purified protein in soluble state rendered it unsuitable for biological studies. Purification of refolded r-bmZPB without urea and devoid of lower molecular weight fragments was achieved by following an alternate methodology that involved purification of inclusion bodies to homogeneity and solubilization in the presence of a low concentration of chaotropic agent (2 M urea) and high pH (pH 12). The solubilized protein was refolded in the presence of oxidized and reduced glutathione. The circular dichroism spectra revealed the presence of both α helical and β sheet components in the secondary structure of the refolded r-bmZPB. The binding of the refolded r-bmZPB to the spermatozoa was evaluated by an indirect immunofluorescence assay and also by direct binding of the biotinylated r-bmZPB. The binding was restricted to the principal segment of the acrosomal cap of capacitated bonnet monkey spermatozoa. In the acrosome-reacted spermatozoa a shift in the binding pattern of r-bmZPB was observed and it bound to the equatorial segment, postacrosomal domain, and midpiece region. Binding of biotinylated r-bmZPB was inhibited by cold r-bmZPB as well as by monoclonal and polyclonal antibodies generated against r-bmZPB. These results suggest that nonglycosylated bmZPB binds to capacitated as well as acrosome-reacted spermatozoa in a nonhuman primate and may have a functional role during fertilization.

fertilization, ovum, sperm

INTRODUCTION

The zona pellucida (ZP), an acellular translucent envelope that surrounds the mammalian oocyte, is composed of three biochemically and immunologically distinct glycoproteins classified as ZP1, ZP2, and ZP3, based on the mobility on SDS-PAGE, and also as ZPA, ZPB, and ZPC, on the basis of the mRNA transcript size, where ZPA is the longest and ZPC is the shortest [1]. The ZP glycoproteins play a crucial role in the initial attachment, followed by tight binding of the spermatozoa to the oocyte in a species-specific manner and in the subsequent complex cascade of events during fertilization [2]. It blocks polyspermy and protects the preimplanted embryo. In the murine model, ZPC acts as a primary receptor for sperm binding to the oocyte and induces the acrosome reaction in the spermatozoa bound to the ZP [3, 4]. ZPA acts as a secondary receptor and maintains the binding of the acrosome-reacted spermatozoa to the ZP [5]. ZPB has been postulated to cross-link a ZPA-ZPC heterodimer. However, in the porcine model, ZPB plays an important role in the gamete interaction as ZPB-ZPC heterocomplexes have been shown to bind with higher affinity to the boar sperm-associated zona receptors, but not free glycoprotein subunits [6]. Similarly, in the rabbit model, both R55 (ZPB) and R45 (ZPC) have been shown to bind to the sperm recombinant Sp17 protein that is expressed in African green monkey kidney cells (COS cells) [7]. Moreover, baculovirus-expressed BV55 (ZPB) has been shown to bind to the sperm in a dose-dependent manner [8]. In view of the existing information in different species, the functional relevance of the individual ZP glycoproteins during fertilization needs further evaluation.

The limiting factor for assessing the functions of the individual ZP glycoproteins during fertilization is the formidable task of obtaining these proteins in reasonable quantities in highly pure form from the native source. To circumvent this, several groups, including ours, have cloned and expressed ZP glycoproteins from various species using a variety of expression systems [4, 8–15]. Purification of the recombinant ZP proteins produced using prokaryotic expression system poses two major hurdles: 1) the recombinant protein corresponding to the full-length transcript is invariably contaminated with the varying amounts of lower molecular weight fragments [11, 12], and 2) renaturation of the purified recombinant zona proteins leads to aggregation [14]. To maintain the solubility of the proteins after purification, a high amount of urea (4–6 M) is required [14], thus making such preparations unsuitable to perform biological studies.

In the present study, we have used bonnet monkey (*Macaca radiata*) ZPB (bmZPB) expressed in *E. coli* as a model protein to address these issues. Attempts have been made to purify the recombinant bmZPB (r-bmZPB) from inclusion bodies without denaturing agents and preferentially devoid of lower molecular weight fragments in biologically active form. The binding of the purified and refolded r-bmZPB to capacitated and acrosome-reacted spermatozoa has also been assessed.

MATERIALS AND METHODS

Expression of r-bmZPB in E. coli

For expression of the bmZPB in pRSET-A vector, (excluding the N-terminal signal sequence and the C-terminus

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transmembrane-like domain) the pBluescript-ZP1 clone (clone 5; [11]) was used as a template for the amplification of the bmZPB (1317 base pair [bp]), by polymerase chain reaction (PCR), using 5′-GGTGAGCTCAAGCCTGAGACACCAGGT-3′ as the forward primer, with a *Sac*I restriction site, and 5′-TCTGGTACCGAGATCAGGACAGGT-3′ reverse primer with a *Kpn*I restriction site. The *Sac*I–*Kpn*I restricted bmZPB insert was ligated downstream of T7 promoter in pRSET-A vector (pRSET-bmZPB; Invitrogen Corp., Carlsbad, CA) in frame with the polyhistidine tag at the N-terminus. The BL21(DE3)pLysS strain of *E. coli*, which is deficient in *omp*T and *lon* proteases, was transformed with the pRSET-bmZPB construct. A positive clone was isolated and checked for expression of r-bmZPB by Western blotting. In brief, the clone was inoculated in 1 ml of Luria Broth (LB; Difco Laboratories, Detroit, MI) containing 100 μg/ml of ampicillin and 25 μg/ml of chloramphenicol and grown overnight at 37°C. The next day cells were subcultured (1:100 dilution) in 1 ml of LB and grown at 37°C until the A_{600} reached a value of approximately 0.5–0.6. The culture was induced with an optimized concentration of 0.5 mM isopropyl-D thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, MO) for 2 h. The cells were centrifuged at 8000 × *g* for 60 sec, and the pellet was analyzed for expression of the r-bmZPB by Western blot.

SDS-PAGE and Western Blot

The cell pellet was boiled for 10 min in 2× sample buffer (0.0625 M Tris pH 6.8, 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.001% bromophenol blue) and resolved on 0.1% SDS 10% polyacrylamide gel [16]. The proteins were either stained with the Coomassie brilliant blue or electrophoretically transferred, overnight, on a 0.45 μm nitrocellulose membrane (BioRad, Hercules, CA). Posttransfer, the membrane was blocked in 50 mM PBS pH 7.4 containing 3% skim milk for 90 min. All subsequent incubations were carried out for 1 h at room temperature. Each incubation was followed by washings with PBS containing 0.1% Tween-20 (PBST). Postblocking, the membrane was incubated with 1:1000 dilution of MA-410 ascites (murine monoclonal antibody generated against porcine ZP3α [ZPB], immunologically cross-reactive with bmZPB; [17]), followed by incubation with horseradish-peroxidase (HRP) conjugated rabbit anti-mouse immunoglobulins (Pierce, Rockford, IL). The blot was developed with 0.6% (w/v) 4-chloro-1-naphthol (Amresco, Solon, OH) in 50 mM PBS containing 25% methanol and 0.06% H_2O_2, followed by extensive washing with MQ water.

Purification of r-bmZPB

In denatured form. To purify the r-bmZPB from the inclusion bodies, a 1000-ml culture was set up at shake-flask level (250-ml culture/flask) and induced with 0.5 mM of IPTG as described above. Cells were pelleted at 1500 × *g* for 30 min at 4°C and the pellet was stored at −70°C until used. The cell pellet (~1 g) was solubilized in 5 ml of buffer A (6 M guanidine HCl, 0.1 M NaH_2PO_4, 0.01 M Tris pH 8.0). The suspension was centrifuged at 10 000 × *g* for 5 min at 4°C and the supernatant containing the recombinant fusion protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen GmbH, Hilden, Germany) as described previously [12]. The eluted protein was concentrated on an Amicon concentrator using YM30 membrane (Amicon Corp., Lexington, MA) and then dialyzed against 100 mM phosphate buffer pH 7.4 containing 4 M urea. The purified protein was quantified by bicinchoninic acid (BCA; Sigma).

Refolded form. Purification of inclusion bodies. To purify the r-bmZPB in renatured form, pRSET-bmZPB clone was grown and induced with IPTG as mentioned above. For purification of the inclusion bodies, the bacterial cell pellet from the 1000-ml culture was resuspended in 10 ml of Tris-HCl buffer (50 mM) pH 8.5 containing EDTA (5 mM) and sonicated for eight cycles (90 sec each; 30 W output; Branson Ultrasonic Corp., Danbury, CT) on ice. Inclusion bodies were collected by centrifugation of the sonicate at 8000 × *g* for 30 min at 4°C. The pellet was washed twice with 15 ml of 50 mM Tris-HCl buffer with 5 mM EDTA containing 2% sodium deoxycholate (Amresco) in order to remove loosely bound *E. coli* proteins from the inclusion bodies [18]. Subsequently, the inclusion body pellet was washed with 50 mM Tris buffer pH 8.5, followed by distilled water to remove detergent. All the buffers used for purification contained 20 mM phenylmethyl sulphonyl fluoride (PMSF; Sigma).

Refolded form. Solubilization of inclusion bodies and refolding of the r-bmZPB. The purified inclusion bodies were solubilized in 100 mM Tris-HCl containing 2 M urea pH 12, at room temperature and centrifuged at 8000 × *g* for 30 min at 4°C. The pH of the solubilized r-bmZPB was immediately brought down to 8.5 by 1 N HCl and extensively dialyzed against renaturing buffer (50 mM Tris buffer pH 8.5, 1 mM EDTA, 0.01 mM reduced glutathione, 0.001 mM oxidized glutathione, and 10% sucrose). The refolded r-bmZPB was further dialyzed against 20 mM Tris pH 8.5, and the protein was estimated using BCA.

Spectroscopic Analysis

The refolded and purified protein was passed through a 0.45 μm filter (Millipore, Bedford, MA) and used for circular dichroism (CD) analysis. The CD spectrum of r-bmZPB was obtained at 25°C in the wavelength range of 190–250 nm using a JASCO-710 spectropolarimeter. The sample was scanned 10 times for data accumulation and the average spectrum was plotted.

Evaluation of Refolded r-bmZPB Binding to Spermatozoa

Indirect immunofluorescence. Semen was obtained from fertile bonnet monkeys (Primate Research Centre, National Institute of Immunology, New Delhi, India) by electroejaculation and liquefied for 30 min at 37°C. The sperm were washed twice in 10 ml of Bigger-Whitten-Whittingham (BWW) medium [19] and centrifuged at 800 × *g*, for 10 min. The pellet was resuspended in BWW (supplemented with 0.3% BSA). The motile sperm were collected by standard swim-up technique, and incubated for 12 h at 37°C in a humidified chamber with 5% CO_2 in air for capacitation. For the preparation of acrosome-reacted spermatozoa, the capacitated and motile sperm were incubated with BWW medium containing calcium ionophore-A23187 (10 μM; Sigma) for 10 min, centrifuged at 800 × *g* for 10 min and resuspended in BWW containing 0.3% BSA. Postcapacitation or after induction of the acrosome reaction, 10^6 sperm were incubated with refolded r-bmZPB or r-bmZPC (20 μg/0.5 ml) for 2 h at 37°C, in a humidified chamber containing 5% CO_2 in air. These sperm binding experiments were repeated three times using semen samples of two different males to rule out any ambiguity.

The sperm were washed thoroughly to remove unbound

r-bmZPB and subsequently, spotted a 10-μl suspension on the multitest microscopic slides, air dried, and fixed in chilled methanol (10 min), and proceeded for analysis by an indirect immunofluorescence assay. The slides were blocked with 3% BSA in PBS at 37°C for 1 h. Slides were washed twice with 50 mM PBS. All subsequent incubations were carried out at 37°C for 1 h, followed by three washings with 50 mM PBS. The slides were incubated with 1:100 dilution of rabbit preimmune serum or rabbit polyclonal antibodies raised against r-bmZPB [11] containing 0.1% BSA. The binding of r-bmZPB was revealed by goat anti-rabbit immunoglobulin-fluorescein isothiocyanate (FITC; Pierce, Rockford, IL) at 1:800 dilution in PBS with 0.1% BSA. The acrosomal status of the spermatozoa was assessed using 10 μg/ml tetramethylrhodamine isothiocyanate (TRITC)-conjugated *Pisum sativum* agglutinin (PSA; it was a kind gift from Dr. Koji Koyama, Department of Obstetrics and Gynaecology, Hyogo College of Medicine, Nishinomiya, Hyogo 663, Japan). Any spermatozoa that demonstrated complete loss of PSA staining in the acrosome or revealed staining at the equatorial region were classified as acrosome reacted. The slides were mounted in glycerol:PBS (9:1) and examined under a fluorescence microscope (Nikon, Chiyoda-ku, Tokyo, Japan).

Using biotinylated r-bmZPB. Direct binding assay. Two milligram of the refolded r-bmZPB, was dialyzed extensively against biotin labeling buffer (0.1 M $NaHCO_3$, 0.1 M NaCl pH 8.0), followed by incubation with NHS-LC-Biotin (Pierce) at 1:20 molar ratio, with end-to-end shaking for 1 h at room temperature. Postincubation, the biotin labeled r-bmZPB was extensively dialyzed against dialysis buffer (0.1 M Tris, 0.2 M NaCl, 0.1% NaN_3 pH 8.0). The concentration of the biotinylated r-bmZPB was estimated by BCA. Capacitated sperm (10^6/0.5 ml) were incubated for 2 h at 37°C in a humidified chamber containing 5% CO_2 in air, with varying concentrations of the biotinylated r-bmZPB (250 ng, 500 ng, and 1 μg). After incubation sperm were washed three times with PBS by centrifugation at 800 × *g* for 10 min. The sperm pellet was resuspended in 0.2 ml HRP-conjugated streptavidin (1:3000 dilution; Pierce) for 1 h at 37°C. Postincubation, sperm were washed three times with PBS and resuspended in 0.1 ml of 0.05% orthophenylenediamine in 50 mM citrate phosphate buffer pH 5.0 with 0.06% hydrogen peroxide as a substrate. The reaction was stopped by 5 N H_2SO_4, and the absorbance of the supernatant was read at 492 nm with 620 nm as a reference filter.

Using biotinylated r-bmZPB. Inhibition assay. Capacitated sperm (10^6/0.5 ml) were incubated with either 250 or 500 ng of the biotinylated r-bmZPB in the presence of 1:100 molar ratio of either cold r-bmZPB or purified monoclonal antibody MA-809, raised against r-bmZPB [17]. In addition, rabbit polyclonal antibodies generated against r-bmZPB as described previously [11] were also used at 1:100 dilution to study the inhibition in the binding of biotinylated r-bmZPB to spermatozoa. Inhibition studies with unlabeled r-bmZPB were carried out by simultaneous addition of biotinylated r-bmZPB and unlabeled r-bmZPB to sperm suspension, whereas, the antibodies were incubated with the biotinylated r-bmZPB for 4 h prior to addition to the sperm suspension.

RESULTS

Expression of r-bmZPB in E. coli and Its Purification Under Denaturing Conditions

Western blot analysis of the host cells transformed with pRSET-bmZPB construct revealed a major band of approximately 55 kDa corresponding to the full-length polypeptide (Fig. 1A), along with prominent lower molecular weight fragments. The r-bmZPB purified using Ni-NTA affinity chromatography appeared as a prominent band of 55 kDa, in addition to lower molecular weight fragments, which however, were present in minute amounts, as analyzed by SDS-PAGE and Western blot (Fig. 1, B and C). The amount of the r-bmZPB purified from pRSET-bmZPB clone, under denaturing conditions, was 6 mg/L in a batch flask culture.

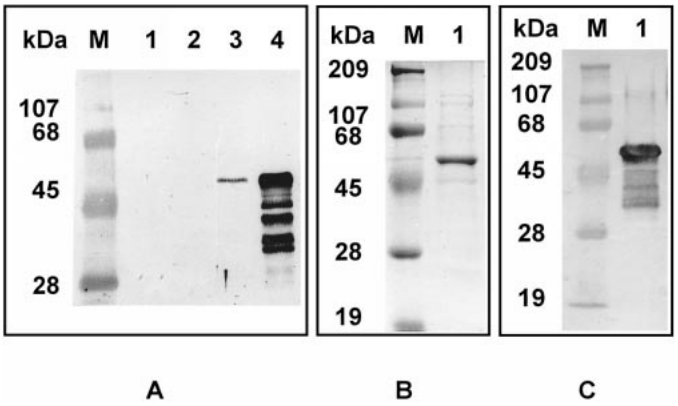

FIG. 1. Electrophoretic and immunoblot analysis of the total cell lysate and r-bmZPB purified under denaturing conditions. BL21(DE3)pLysS cells transformed with pRSET-bmZPB construct was grown in LB medium and induced with IPTG for expression of the r-bmZPB. Panel **A** shows the pattern of expressed r-bmZPB as revealed by Western blot analysis. Various lanes represent M, molecular weight markers; lanes 1 and 2, uninduced and induced wild-type cells; lanes 3 and 4, uninduced and induced cells harboring the pRSET-bmZPB. Panels **B** and **C** represent the respective Coomassie-stained gel (25 μg/lane) and Western blot (5 μg/lane) of r-bmZPB purified using Ni-NTA affinity matrix. Lanes are represented as M, molecular weight markers; lane 1, Ni-NTA purified protein.

Refolded r-bmZPB and CD Spectra

The r-bmZPB, purified using an alternate method, resolved as a single band of 55 kDa on SDS-PAGE and Western blot (Fig. 2, A and B). Purification of the r-bmZPB by this method not only yielded the protein in refolded form but also resolved the problem of contamination with lower molecular weight fragments to a great extent. However, the

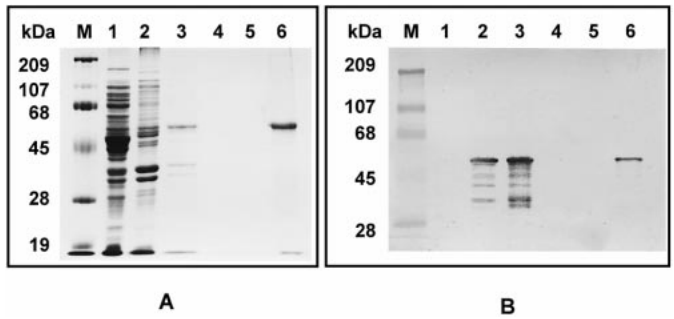

FIG. 2. SDS-PAGE and immunoblot of the purified and refolded r-bmZPB. The r-bmZPB from BL21(DE3)pLysS *E. coli* cells harboring pRSET-bmZPB plasmid was purified in renatured conditions as described in *Materials and Methods.* The samples obtained at various steps of purification were resolved on a 0.1% SDS 10% PAGE, electrophoretically transferred on a nitrocellulose membrane and probed with MA-410. Panel **A** represents Coomassie-stained gel, **B**) Western blot of the same. Lanes: M, molecular weight markers; lane 1, supernatant after the sonication; lanes 2 and 3, deoxycholate washes; lane 4, Tris wash; lane 5, distilled water wash; lane 6, purified and refolded r-bmZPB.

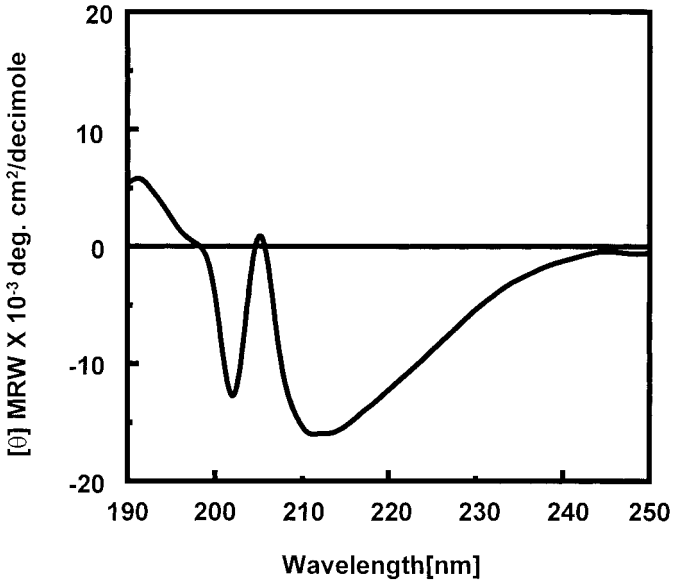

FIG. 3. Circular dichroism of the purified and refolded r-bmZPB. Spectra of the purified and refolded r-bmZPB (50 μg/ml) from pRSET-bmZPB clone in 20 mM Tris buffer pH 8.5, was scanned through the far UV range (190–250 nm).

yield of the r-bmZPB purified by this method was 3.2 mg/L of culture as compared to 6 mg/L when the purification was done under denaturing conditions from the same clone.

The CD spectrum of the r-bmZPB at 190–250 nm showed a minimum at 210–215 nm, implying the presence of both α helical and β sheet components in the refolded r-bmZPB (Fig. 3).

r-bmZPB Binds to the Spermatozoa

The typical binding pattern of the r-bmZPB to the spermatozoa is shown in Figure 4. After capacitation, 76.00% ± 8.38% (mean ± SD) sperm showed binding of TRITC-PSA. In the double staining experiments, from among those binding TRITC-PSA, 77.00% ± 16.29% sperm showed the binding of r-bmZPB. The binding of r-bmZPB was localized to the principal segment of the acrosomal cap of the capacitated spermatozoa (Fig. 4C). However, there was no binding seen when the preimmune rabbit sera was used instead of the rabbit polyclonal antibodies (data not shown). Sperm incubated with r-bmZPC expressed in *E. coli* and probed with immune sera raised against r-bmZPB also failed to show any fluorescence (data not shown). The acrosomal status of the spermatozoa was assessed using TRITC-PSA conjugate (Fig. 4, B and E). Any spermatozoa demonstrating complete loss of the TRITC-PSA staining or the staining limited to the equatorial region was classified as acrosome-reacted spermatozoa (Fig. 4E). The A23187 ionophore-induced acrosome exocytosis was found in a proportion of 78.38% ± 11.45% and r-bmZPB showed a differential shift in the binding pattern in 79.00% ± 12.28% of the acrosome-reacted spermatozoa. The r-bmZPB was now located in the equatorial segment, postacrosomal domain, and midpiece region (Fig. 4F).

The binding of the purified and refolded r-bmZPB to the spermatozoa was also ascertained using biotinylated

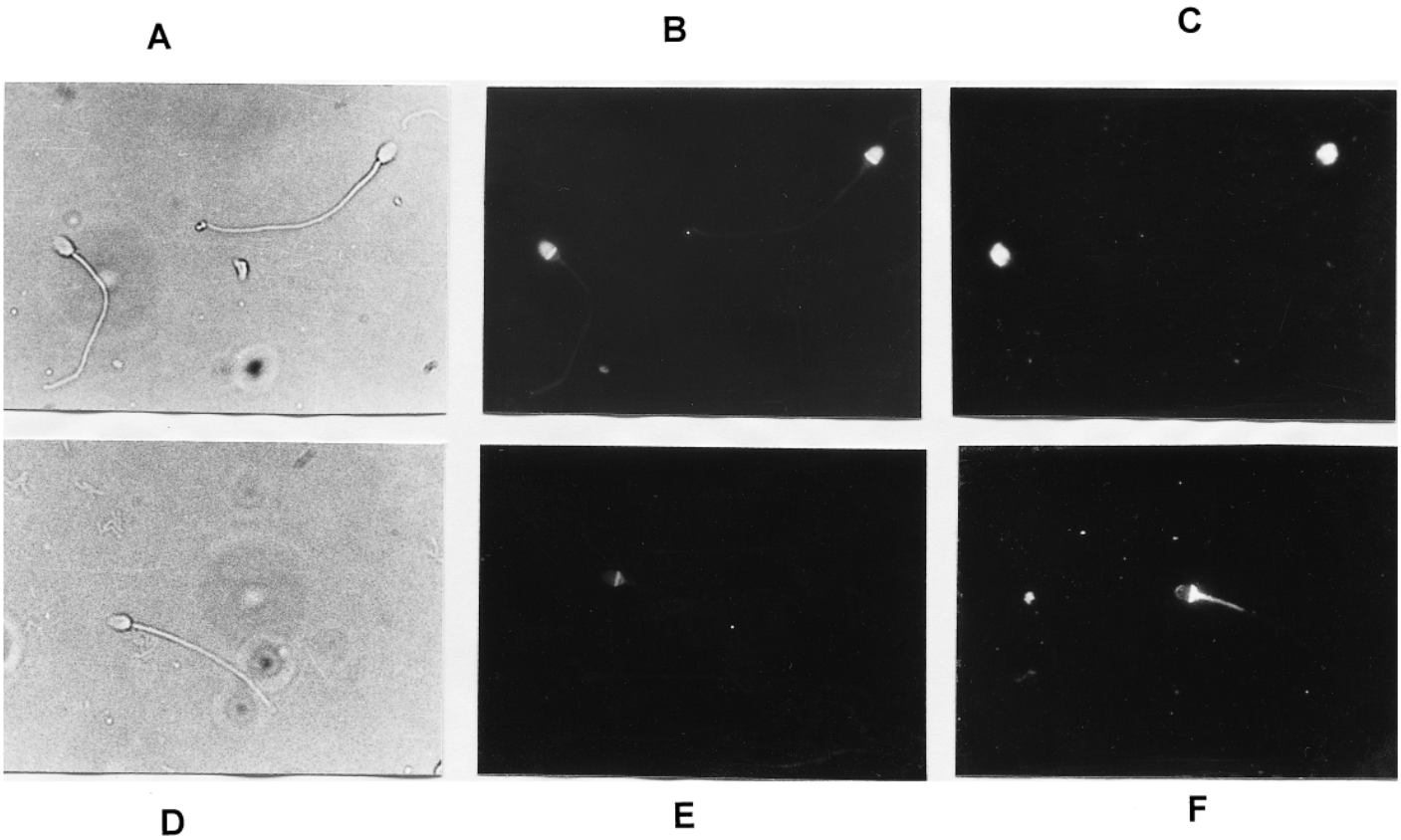

FIG. 4. Analysis of the binding of r-bmZPB to the spermatozoa by indirect immunofluorescence. The capacitated or acrosome-reacted sperm (10^6/0.5 ml) were incubated with purified and refolded r-bmZPB (40 μg/ml) for 2 h and processed for immunofluorescence as described in *Materials and Methods.* The upper panel of microphotographs (×400) represent capacitated sperm; lower panel, acrosome-reacted spermatozoa. **A** and **D**) Phase contrast, **B** and **E**) TRITC-PSA staining, **C** and **F**) Rabbit polyclonal antibodies against r-bmZPB; FITC staining.

TABLE 1. Binding of the refolded biotinylated r-bmZPB to the bonnet monkey spermatozoa in absence or presence of r-bmZPB, monoclonal antibody, and polyclonal antibodies against r-bmZPB.

Concentration of biotinylated r-bmZPB (ng/0.5 ml)	Concentration of ligand used for inhibition	Mean absorbance at 492 nm ± SEM
0	—	0.26 ± .004
250	—	1.72 ± .038
500	—	2.72 ± .062
1000	—	3.19 ± .058
250	1:100; rbmZPB[a]	1.23 ± .063 ($P < 0.0025$)
500	1:100; r-bmZPB[a]	1.73 ± .026 ($P < 0.0001$)
250	1:100; MA-809[a]	0.40 ± .008 ($P < 0.0001$)
500	1:100; MA-809[a]	0.44 ± .012 ($P < 0.0001$)
250	1:100; RPA[b]	0.21 ± .007 ($P < 0.0001$)
500	1:100; RPA[b]	0.29 ± .012 ($P < 0.0001$)

[a] Expressed in molar ratio.
[b] Expressed as dilution; RPA, rabbit polyclonal antibodies raised against r-bmZPB [11].

r-bmZPB. The results are summarized in Table 1. A dose-dependent increase in the binding of biotinylated r-bmZPB to sperm was observed. The binding of the biotinylated r-bmZPB to the capacitated sperm was found to be inhibited in the presence of 100-fold concentration of the cold r-bmZPB ($P < 0.0001$). Prior incubation of the r-bmZPB with the monoclonal antibody, MA-809, or rabbit polyclonal antibodies generated against r-bmZPB, also significantly inhibited its binding to the sperm.

DISCUSSION

Several groups have reported generation of multiple gene products during the heterologous expression of proteins in *E. coli* [15, 20]. The reason or reasons for the presence of lower molecular weight fragments remains elusive at the moment, but could arise due to 1) multiple initiation sites in mRNA, 2) premature translation termination, 3) specific or nonspecific proteolysis of the full-length polypeptide, and 4) a combination of these. In order to minimize the presence of such truncated fragments, we opted to clone bmZPB in the pRSET vector and express it in *lon* and *omp*T protease-deficient host strain (BL21[DE3]pLysS) of *E. coli*. The Ni-NTA purified r-bmZPB from this clone resolved largely as a single band, though minor amounts of truncated fragments were also visible, which were present in far less amounts compared to the r-bmZPB expressed in pQE30 vector (Fig. 1, B and C; [11]). The strategy to purify only the full-length polypeptide corresponding to the transcript of r-bmZPB, using His_6 at the C-terminus, did not eliminate the truncated fragments (data not shown). Proteolytic degradation during expression of the r-bmZPB could generate the truncated polypeptide possessing His_6 tag, which can bind to the Ni-NTA resin and copurify along with the full-length r-bmZPB. In Ni-NTA only two of the six ligand binding sites in the coordination sphere of the Ni ion are free to interact with the histidine and the presence of two or more histidines in close proximity on their surface is sufficient for the interaction with Ni-NTA. The presence of 12 histidine residues in the deduced amino acid sequence of bmZPB may also contribute for the presence of truncated fragments in the Ni-NTA affinity-purified protein. The presence of a prominent 107-kDa band in SDS-PAGE but its absence from the Western blot in the Ni-NTA purified protein suggests the copurification of some of the host proteins (Fig. 1, B and C) along with the r-bmZPB. This could be due to its being histidine-rich or interaction with the carbohydrate moieties of the Ni-NTA resin.

The purification of the full-length r-bmZPB in refolded form was achieved by employing a different procedure, directly from the inclusion bodies, as the formation of inclusion bodies takes place due to intramolecular hydrophobic interactions between a single type of molecules [21]. Moreover, the presence of truncated products, as observed during purification of r-bmZPB under denaturing conditions, was alleviated to a great extent as the proteins in the inclusion bodies are resistant to the proteolytic degradation, due to the acquired compact conformation [22]. The purification of recombinant protein in biologically active form was facilitated by the fact that the native-like secondary conformations are preserved in the inclusion bodies [23, 24]. Destabilization of the aggregates in the inclusion bodies, at high alkaline pH and solubilization of r-bmZPB in presence of mild chaotropic agent (2 M urea), may be attributed to the change in charge distribution over the polypeptide chain, which in turn, reduces the hydrophobic and ionic interactions [25, 26]. Subsequent to solubilization of the purified inclusion bodies containing r-bmZPB, refolding was achieved by its dialysis against the buffer containing reduced and oxidized forms of glutathione, which may be assisting in stabilization of the 19 cysteine residues present in r-bmZPB, through enhanced thiol-disulfide exchange and by restricting the conformational flexibility of the unfolded state [27]. The secondary structure of refolded r-bmZPB looks like that of α helical and β sheet conformation, as suggested by a single, broad minimum skewed toward 210 nm than the 222-nm band.

The availability of the purified and refolded r-bmZPB expressed in *E. coli* provided a homogeneous molecular population free from glycosylation for experimental evaluation of its interaction with the sperm. In this study, r-bmZPB is shown to bind to the principal segment of the capacitated spermatozoa. Failure of binding of r-bmZPB to 22.47% ± 16.29% (mean ± SD) of the capacitated sperm that bound PSA may represent a population of spermatozoa that failed to capacitate. A profound change in the binding pattern was observed to the acrosome-reacted spermatozoa, which was now relocated to the equatorial segment, postacrosomal region, and the midpiece. Similar observations have been made earlier in rabbit model with the glycosylated recombinant ZPB expressed using the baculovirus expression system [8]. The observed shift in the binding pattern of r-bmZPB could be due to the reorientation of the bmZPB receptors in the equatorial, postacrosomal region, and midpiece of the acrosome-reacted spermatozoa. The principal segment of the acrosomal cap has been proposed to be involved in the initial binding of the spermatozoa to the ZP and the equatorial segment is responsible for initiating the fusion of the sperm membrane with the oolema [28, 29]. The changes in binding pattern of r-bmZPB to the spermatozoa following acrosome reaction suggest that it may be involved in initial recognition as well as postrecognition events during fertilization. The binding was reconfirmed by employing biotinylated r-bmZPB. The unlabeled r-bmZPB could inhibit the binding of the biotinylated protein. Moreover, inhibition in the binding of biotinylated r-bmZPB to the spermatozoa in the presence of MA-809 and rabbit polyclonal antibodies raised against r-bmZPB,

further confirms the specificity of the r-bmZPB binding (Table 1).

It has been suggested that the sperm-oocyte interaction in mammals is mediated by the carbohydrate moieties present on the sperm-adhesive glycoproteins within the zona matrix and complementary zona-binding proteins on the spermatozoon's surface [28]. Loss of the receptor activity of the ZPC on removal of *O*-linked oligosaccharides and not *N*-linked oligosaccharides and the ability of a specific class of ZPC-derived *O*-linked oligosaccharides (~3900 M_r) to exhibit receptor activity in vitro strongly suggests the role of carbohydrates in the oocyte-sperm interaction [30]. However, binding of recombinant human ZPC expressed in *E. coli* to human spermatozoa and its ability to induce the acrosome reaction suggests a significant role for the polypeptide backbone [20]. Monoclonal antibodies (MA-809, -811, -813, and -825) raised against r-bmZPB expressed in *E. coli* and recognizing an epitopic motif ''DAPDTDWCDSIP'' inhibited human sperm-egg binding in vitro, as shown previously [17]. These observation also suggested the role of the polypeptide backbone of the bmZPB in sperm binding to the oocyte. This study demonstrates for the first time the ability of nonglycosylated r-bmZPB to bind to the spermatozoa.

In conclusion, we have been able to eliminate the problem of truncated gene products during expression of the bmZPB, in *E. coli*, and to refold it. We report for the first time the binding of *E. coli*-expressed r-bmZPB to the capacitated as well as acrosome-reacted spermatozoa. Further studies will be required to precisely define its role during various steps of fertilization and relevance of carbohydrate moieties of ZPB in this process.

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