RESEARCH

Production of Tag-Free Recombinant Fusion Protein Encompassing Promiscuous T Cell Epitope of Tetanus Toxoid and Dog Zona Pellucida Glycoprotein-3 for Contraceptive Vaccine Development

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Abstract Affinity tags can interfere in various physico-chemical properties and immunogenicity of the recombinant proteins. In the present study, tag-free recombinant fusion protein encompassing promiscuous T cell epitope of tetanus toxoid [TT; amino acid (aa) residues 830-844] followed by dilysine linker and dog zona pellucida glycoprotein-3 (ZP3; aa residues 23-348) (TT–KK–ZP3) was expressed in *Escherichia coli*. The recombinant protein, expressed as inclusion bodies (IBs), was purified by isolation of IBs, processed to remove host cell proteins, followed by solubilization and refolding. A specific 39 kDa protein including ZP3 was identified by SDS-PAGE. CD spectra showed the presence of α-helices and β-sheets, and fluorescent spectroscopy revealed emission maxima of 265 A.U. at 339 nm for refolded protein and showed red shift in the presence of 6 M guanidine hydrochloride. Immunization of inbred FvB/J female mice with purified recombinant TT–KK–ZP3 (25 μg/animal) led to generation of high antibody titers against the recombinant protein. The antibodies reacted specifically with ZP matrix surrounding mouse oocytes. Immunized mice showed significant reduction in fertility as compared to the control group. The studies described herein provide a simple method to produce and purify tag-free recombinant protein for the development of a contraceptive vaccine.

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Introduction

Zona pellucida (ZP) glycoproteins by virtue of their critical role during fertilization have been proposed as candidate immunogens for contraceptive vaccines [7, 8]. Contraceptive vaccines based on native porcine ZP have been used for the management of feral horses (*Equus caballus*) and white-tailed deer (*Odocoileus virginianus*) populations [15, 24]. Immunization of female dogs with either crude porcine ZP or purified porcine ZP along with different adjuvants also led to curtailment of infertility [22]. Infertility was associated with prolonged pro-estrus and estrus cycles accompanied by abnormal estradiol and progesterone levels. Ovarian histology of the immunized bitches revealed disturbances in the follicular development; granulosa cell nests in crude porcine ZP immunized group and luteal cysts in those immunized with purified porcine ZP [23]. To overcome the limited availability of native ZP glycoproteins, contraceptive vaccines based on recombinant zona proteins to control the population of marsupials [4, 16] and street dogs [35] have been proposed. The immunogen employed in the proposed vaccine for curtailing fertility of female dogs was *Escherichia coli*-expressed recombinant dog zona pellucida glycoprotein-3 (ZP3) with His$_6$-tag and chemically conjugated to diphtheria toxoid (DT) used as carrier protein to elicit antibody response in a homologous model [35]. It is difficult to produce batches of vaccine based on chemical conjugation with reproducible quality control. Translation fusion of recombinant antigen and carrier protein provides a viable alternative to the chemical conjugation process. Further, inclusion of His$_6$-tag in the

expressed recombinant protein may have significant consequences with respect to expression levels [36, 39], solubility of protein [2, 39], protein folding [17], stability [18], and disulfide bonding patterns [17]. The presence of His$_6$-tag may also influence the efficacy of the vaccine as demonstrated in the case of malaria infection. Antibodies generated against His$_6$-tag-free recombinant *Plasmodium falciparum* MSP1 have higher percent parasite growth inhibition as compared to the antibodies generated against His$_6$-tagged MSP1 [14]. Even though tags can be removed by proteolytic cleavage, it is difficult to produce 100 % tag-free recombinant protein [19]. Therefore, to overcome problems related to affinity tags, there is an urgent need to develop methods for the production and purification of the tag-free recombinant proteins, which can be used for immunization purpose.

Bacterial inclusion bodies (IBs) can prove to be a boon in overcoming the bottleneck of recombinant protein purification without any affinity tag [5]. In *E. coli*, IBs are largely formed from specific aggregates of over-expressed recombinant protein [26, 34]. The major advantage of using recombinant proteins produced as IBs is the native-like secondary structure of the expressed protein and resistance to proteolytic degradation due to compact conformation [37]. Further, it has been reported that IBs are formed mainly due to the specific molecular interactions among a single type of protein molecules, and hence, represent a highly pure protein deposit of the target recombinant protein [34, 38]. Solubilization of IBs has a direct impact on refolding of the denatured proteins and hence, efforts should be made to minimize the use of harsh conditions for solubilization [28]. The process of renaturation needs to be optimized based on the method of solubilization with minimum possible aggregation of the protein [3].

In the present study, to avoid chemical conjugation of *E. coli*-expressed recombinant dog ZP3 with DT, cDNA encoding promiscuous T cell epitope of tetanus toxoid (TT) corresponding to amino acid (aa) residues 830-844 was fused at the N terminus of cDNA encoding dog ZP3, devoid of putative signal peptide and transmembrane-like domain following furin cleavage site (aa residues 23-348), separated by dilysine linker (TT–KK–ZP3). The recombinant fusion protein was expressed as IBs in *E. coli* and subsequently refolded and purified. The immunogenicity and contraceptive efficacy of the purified protein has been evaluated in female mice.

Materials and Methods

Cloning and Expression of Fusion Protein Encompassing Promiscuous T Cell Epitope of TT and Dog ZP3

To have cDNA encoding TT–KK–ZP3 comprising promiscuous T cell epitope of TT, dilysine linker and canine ZP3 that is devoid of putative signal peptide and transmembrane-like domain following furin cleavage site, forward primer incorporating promiscuous T cell epitope of TT (830-844 aa), dilysine linker and 6 aa of dog ZP3 (aa residues 23-28) with *Bam*HI restriction enzyme site (5′-CG*GGATCC*GCACAGTATATAAAAGCAAATTCT AAATTTATAGGTATAACTGAACTAAAAAAACAGA CCATCTGGCCAACT-3′) and reverse primer incorporating a stop codon with *Bgl*II restriction site (5′-AAA*A GATCT*TTAA GTGTGGGAAACAGACCT -3′) were designed. The PCR amplification was performed in 50 µl final reaction volume with Taq DNA polymerase, 10 ng of dog ZP3 DNA present in pQE-30 vector [33] as template, and 50 pmol of primers. PCR amplification cycles involved initial denaturation at 94 °C for 10 min and 25 cycles of 94 °C for 30 s, 62 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR amplified fragment was ligated in pGEMT-Easy cloning vector (Promega Corporation, Madison, WI, USA) followed by pQE-60 expression vector (QIA Express; Qiagen GmbH, Hilden, Germany) under T5 promoter-lac operator control. The resultant plasmid DNA was used to transform *E. coli* M15 (pREP4) host cells for the expression of TT–KK–ZP3. To check expression of the recombinant protein, the transformants positive for the presence of insert were inoculated into 3 ml of Luria broth (LB) medium (Difco Laboratories, Detroit, MI, USA) containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) and grown overnight at 37 °C in a shaker incubator. The cultures were diluted 1:100 into fresh medium and grown until the cell density reached an absorbance at 600 nm (A$_{600}$) of approximately 0.5–0.6. Expression of the recombinant protein was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 3 h. Uninduced transformed cells were also grown as negative control. The cells were centrifuged at 6,500×*g* for 5 min and the resulting pellets were stored at −20 °C until used. Conditions were optimized for the expression of TT–KK–ZP3 with respect to time and concentration of IPTG used for induction.

SDS-PAGE and Immunoblotting

The cell pellet obtained from 1 ml culture was boiled for 10 min in 5× sample buffer [0.15 M Tris–HCl, pH 6.8, 5 % sodium dodecyl sulfate (SDS), 25 % glycerol, 12.5 % β-mercaptoethanol, 0.025 % bromophenol blue] and proteins were resolved by 0.1 % SDS-10 % PAGE and processed for Western blot analysis essentially as described previously [33]. Expression of the recombinant protein was detected by mouse monoclonal antibody (MAb); MA-451 [1].

Localization of TT–KK–ZP3

Cells were harvested from 50 ml of induced culture by centrifugation at $6,500\times g$ for 15 min at 4 °C. Pellet was resuspended in 5 ml of sonication buffer (50 mM phosphate pH 7.4, 300 mM NaCl). The cells were lysed by sonication using a Branson Sonifier-450 for 3–4 cycles of 45 s each (30 Watt output; Branson Ultrasonic Corp., Danbury, CT, USA) and centrifuged at $12,000\times g$ for 20 min at 4 °C. The supernatant and the cell pellet representing the soluble and insoluble fractions were analyzed by SDS-PAGE and Western blotting as described above.

Isolation and Purification of IBs

For purification of recombinant TT–KK–ZP3, culture was grown at a shake-flask level (250 ml culture/flask; total volume 1 l). The cells were induced with optimized concentration of IPTG (0.3 mM) for 6 h at 37 °C followed by centrifugation at $6,500\times g$ for 30 min at 4 °C. For purification of IBs, the cell pellet (1 g) was resuspended in 10 ml Tris buffer (50 mM; pH 8.0) containing 5 mM EDTA and sonicated using Branson sonifer-450 for 8–10 cycles each of 90 s at a 40 watt output (with 2 min pause intervals) on ice. The IBs were collected by centrifugation at $8,000\times g$ for 30 min at 4 °C. Various reagents were tested for their ability to release host proteins from the IBs while leaving the heterologous protein insoluble. In 50 mM Tris buffer, 5 mM EDTA, 2 mM DTT, pH 8.0; three different detergent concentrations were tested including (i) no detergent, (ii) 1 % sodium deoxycholate (Amresco, Solon, Ohio, USA), (iii) 2 % sodium deoxycholate, and (iv) 2 % sodium deoxycholate and 100 mM NaCl. Four identical IBs pellets (equivalent of 250 ml culture volume) were suspended in 15 ml of each of these solutions, incubated for 20 min at RT followed by centrifugation and the same process was repeated one more time. Subsequently, all four IBs pellets were washed with 50 mM Tris buffer (pH 8.0), followed by final washing with double distilled water.

Solubilization of IBs and Refolding of the Recombinant Protein

The purified IBs thus obtained were solubilized in 5 ml of 100 mM citrate buffer, pH 4.0 containing one of the following (i) 8 M urea, (ii) 8 M urea and 5 mM β-mercaptoethanol, and (iii) 8 M urea and 10 mM β-mercaptoethanol, for 4–6 h at RT with end to end mixing. Subsequently, the samples were centrifuged at $12,000\times g$ for 30 min at 4 °C and the supernatant containing the recombinant protein and the insoluble fractions thus obtained were analyzed by SDS-PAGE. Further, to optimize the refolding process and to recover maximum amount of protein, two different methods

of pulsatile dilution of the protein were tried. In one of the procedures, solubilized recombinant TT–KK–ZP3 was diluted tenfold by adding drop wise ($\sim$200 μl/min) 100 mM citrate buffer, pH 4.0 to the protein (Sample 1) with gentle stirring at 4 °C. Alternatively, solubilized protein was added dropwise ($\sim$200 μl/min) into 100 mM citrate buffer, pH 4.0 to achieve tenfold dilutions (Sample 2). The refolded proteins were dialyzed extensively against 20 mM Tris–HCl buffer, pH 6.8 followed by centrifugation at $12,000\times g$ for 30 min at 4 °C to remove any aggregated particles. Protein concentration of both the samples was determined using BCA estimation kit (Pierce, Rockford, IL, USA).

Fluorescence Spectroscopy Studies

Fluorescence spectra of protein renatured with the above two methods and the protein sample denatured by adding 6 M guanidine hydrochloride (Control) were recorded at 25 °C on a Cary Eclipse Spectrofluorometer (Varian, Mulgrave, Victoria, Australia). Typically 200 μg/ml protein in 20 mM Tris–HCl, pH 6.8, was used and the fluorescence emission spectra were recorded from 300 to 400 nm upon excitation at 280 nm. The excitation and emission slit widths were kept at 2 and 5 nm, respectively. All fluorescence spectra were normalized and corrected for buffer contributions.

Circular Dichroism (CD)

The Far UV CD spectra of refolded recombinant TT–KK–ZP3 (Sample 1) was recorded at 25 °C on a Jasco 700 spectro-polarimeter by pipetting 200 μl of the protein sample (300 μg/ml) in the cuvette of 1 mm path length. To validate the presence of secondary structure in the refolded protein, the same concentration of the protein was allowed to denature by addition of 6 M guanidine hydrochloride (Control). Replicate spectra were averaged and blanked against 20 mM Tris–HCl buffer, pH 6.8.

Immunization Studies

Inbred FvB/J female mice were used for active immunization with recombinant TT–KK–ZP3 as they have higher litter size as compared to BALB/cJ. Mice, 8–10 weeks of age, kept under the conventional containment levels at the Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India, were used. These studies were conducted as per the guidelines and approval of the Institutional Animals Ethics Committee. A group of female inbred FvB/J mice ($n = 10$) was immunized subcutaneously with 25 μg of recombinant TT–KK–ZP3 protein per animal with 5 % Montanide™ PetGel A (Seppic, Paris, France) [29] as an adjuvant. A control group of female mice ($n = 10$) was injected with the equivalent amount of

the adjuvant only. Primary immunization was followed by two booster doses of the same amount of recombinant protein, administered intraperitoneally on days 21 and 35.

Determination of Antibody Titre by Enzyme Linked Immunosorbent Assay (ELISA)

Animals were bled retro-orbitally on days 0, 20, 34, and 49. Serum samples were processed for determination of antibody titer against recombinant TT–KK–ZP3 by ELISA essentially as described previously [12]. In brief, microtitration plates (Nunc a/s, Roskilde, Denmark) were coated with the recombinant TT–KK–ZP3 (200 ng/well) in 0.2 M carbonate buffer, pH 9.5 at 37 °C for 1 h and then at 4 °C overnight. The plates were washed once with PBS and incubated with 1 % BSA in PBS (200 μl/well) for 2 h at 37 °C for blocking the non-specific sites. Post-blocking, the plates were incubated with doubling dilutions of serum samples (100 μl/well) collected at different time points for 1 h at 37 °C. The plates were washed three times with PBS containing 0.05 % Tween-20 (PBST) followed by incubation for 1 h at 37 °C with the optimized dilutions in PBST of any one of the following secondary antibody-enzyme conjugates: (i) goat anti-mouse IgG (H + L) conjugated to horseradish peroxidase (HRP; Pierce Biotechnology, Rockford, IL, USA), (ii) goat anti-mouse IgG_1-HRP conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or (iii) goat anti-mouse IgG_{2a}-HRP conjugate (Santa Cruz Biotechnology, Inc.). Following three washes with PBST, plates were incubated with 0.05 % orthophenylenediamine in 50 mM citrate phosphate buffer, pH 5.2, with 0.06 % hydrogen peroxide as the substrate to develop color. The reaction was stopped with 50 μl of 5 N H_2SO_4 and absorbance read at 490 nm with 630 nm as reference filter. Values are represented as antibody titers, which were expressed as antibody units (AU), i.e., reciprocal of the dilution of the serum giving an absorbance of 1.0.

Reactivity of Antibodies with Mouse Oocytes by Indirect Immunofluorescence

FvB/J female mice (4 week old) were super-ovulated by injecting intraperitoneally PMSG (5 IU/mice; Intervet, Boxmeer, Netherlands) followed by hCG (5 IU/mice; Intervet India Pvt. Ltd.) after 48 h. The next day, animals were euthanized by cervical dislocation and ovaries were removed surgically. Oocytes retrieved from Ampulla region were washed once with Brister modified oocyte culture (BMOC) medium (Gibco, Grand Island, NY, USA) followed by treatment for 5 min with hyaluronidase (50 μg/ml) at room temperature to obtain denuded oocytes. The oocytes were subsequently incubated with 50 mM PBS, pH 7.4 supplemented with 1 % BSA for 45 min at 37 °C. The oocytes

($n = 5$) were washed twice with PBS followed by incubation with 1:20 dilution of pre-immune and immune mouse serum samples for 45 min at 37 °C. After washing four times with PBS, oocytes were further incubated with 1:400 dilutions of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Alexa Fluor® 488; Invitrogen, Carlsbad, CA, USA) for 45 min at 37 °C. After several washes in PBS, the oocytes were observed under fluorescence microscope (Nikon Optiphot; Nikon Corp., Tokyo, Japan).

Fertility Analysis of the Immunized Animals

Immunized female mice comprising the above groups were mated with the healthy male mice of proven fertility 15 days after the third injection. Mating was performed by housing two immunized female mice along with a male mouse per cage. Male mice were rotated every third day. The male and female mice were co-habitated for 15 consecutive days to assess the contraceptive efficacy.

Statistical Analysis

The statistical significance for the contraceptive efficacy and pups/litter of the group of mice immunized with the recombinant protein as compared to the control group was determined by Students' t test. A p value of <0.05 was considered to be statistically significant.

Results and Discussion

The recombinant ZP glycoproteins have been proposed as the candidate antigens for the development of immunocontraceptive vaccines. Although the His_6-tag aids in the purification of recombinant proteins, it can create unintended problems related to protein solubility, susceptibility to aggregation, and immunogenicity [14]. Hence, to overcome these problems, we hereby cloned and expressed in E. coli a fusion protein (TT–KK–ZP3) encompassing promiscuous T cell epitope of TT (aa residues 830-844) followed by two lysine residues linker and dog ZP3 (aa residues 23-348) devoid of putative signal peptide and transmembrane-like domain without any affinity tag. This avoids chemical conjugation of dog ZP3 with carrier protein, which otherwise is required to elicit good immune response in homologous animal models. Dilysine linker is the target site for lysosomal protease cathepsin B, which is an important protease in the context of MHC II antigen presentation [21]. The successful cleavage at dilysine linker site by cathepsin B ensures generation of antibodies against the fusion partners separately, concurrently avoiding the generation of antibodies against the aa sequence which arises due to fusion of fragments via linker [27].

Cloning and Expression of Recombinant TT–KK–ZP3 in *E. coli*

The DNA encoding TT–KK–ZP3 was PCR amplified (∼1.0 kb) using plasmid DNA encoding dog ZP3 (aa residues 23-348) as template and subsequently cloned in pQE-60 expression vector. Design of the construct encoding TT–KK–ZP3 in pQE-60 expression vector has been shown in Fig. 1a. The M15 (pREP4) *E. coli* cells harboring the plasmid encoding TT–KK–ZP3 when induced with 1 mM IPTG revealed a major band corresponding to ∼39 kDa (Fig. 1b) by Western blot. In addition, a minor band of ∼34 kDa was also observed. No specific band was, however, observed in the non-induced cells. The cellular localization of TT–KK–ZP3 revealed that the recombinant protein was present only in the insoluble intracellular fraction as IBs (Fig. 1c). This may be due to the over-expression of the recombinant protein resulting in the formation of IBs. Additionally, the lack of post-translational modifications in *E. coli* has also been suggested to contribute to aggregation [9, 12]. The optimum expression of TT–KK–ZP3 was obtained when cells were induced for 6 h (Fig. 2a). Induction with 0.3–1.0 mM IPTG exhibited the same level of protein expression (Fig. 2b) as depicted by Western blot analysis. Therefore, 0.3 mM IPTG concentration was used in all the subsequent experiments.
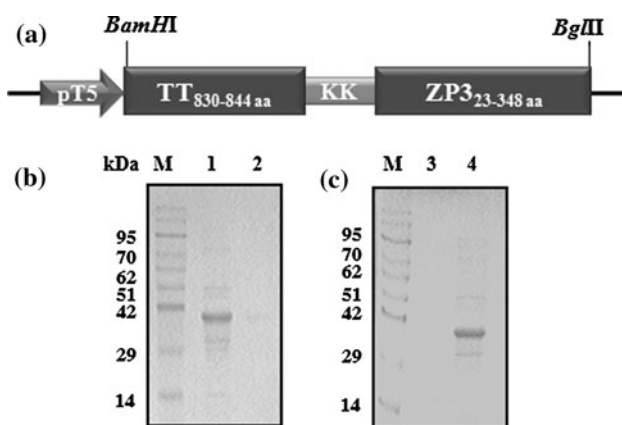

Fig. 1 Construct design and immunoblot analysis of *E. coli*-expressed TT–KK–ZP3. **a** Shows schematic diagram of the construct design representing dog ZP3 (aa residues 23-348) which is devoid of putative signal peptide and transmembrane-like domain after furin cleavage site was linked to T cell epitope of TT by dilysine linker at N terminus in pQE-60 expression vector. **b** Shows Western blot of *E. coli* M15 (pREP4) cells harboring TT–KK–ZP3 in pQE-60 vector induced with 1 mM IPTG for 3 h at 37 °C; *lanes 1* and *2*: induced and uninduced cells harboring plasmid DNA encoding TT–KK–ZP3, respectively. **c** Shows Western blot of localization of expressed TT–KK–ZP3; *lanes 3* and *4* represent soluble and insoluble intracellular fractions of induced cells harboring plasmid DNA encoding TT–KK–ZP3. *Lane M* represents molecular weight markers

Use of High Concentration of Chaotropic Agents for Optimal Solubilization of IBs

Inclusion bodies were isolated from the bacterial cell pellet after sonication and purified using different buffer conditions. The maximum recovery of the recombinant protein with minimum host cell proteins was obtained by washing the IBs in 50 mM Tris (pH 8.0), 5 mM EDTA containing 2 % sodium deoxycholate, 2 mM DTT, and 100 mM NaCl. Sodium deoxycholate is a known surfactant which helps in solubilization of membrane debris and also helps to remove the non-specifically bound contaminants from IBs [11]. Addition of 100 mM NaCl is found to have increased the purity of IBs. Purified IBs were then solubilized in various denaturing buffers. When equal amount of IBs were treated with either 100 mM citrate buffer supplemented with 8 M urea, pH 4.0 or the same buffer further supplemented with 5 or 10 mM β-mercaptoethanol, 10 mM β-mercaptoethanol aided in greater degree of solubilization of IBs (Fig. 3a). A dominant band is present at ∼39 kDa and a very minor band, if present, at ∼34 kDa. Being a reducing agent and hence a denaturant, it was expected that β-mercaptoethanol would aid in solubilization of IBs by disrupting the disulfide bonds present in IB proteins. Usually, the activity of the reducing agents is inhibited at low pH (typically, below 8), where the protonated thiol form is favored relative to deprotonated thiolate form and only thiolate group of the reducing agent is able to attack sulfur atom of the disulfide bond and not the protonated thiol form. Improved solubilization of IBs at low pH in the presence of β-mercaptoethanol suggested that ionic interactions contributed more significantly than the mixed disulfide bonds in IBs of fusion protein. More than 95 % pure protein (Fig. 3b), in which the minor band at ∼34 kDa has been reduced to a negligible level, was obtained by following the above procedure. Experiments were repeated 2–3 times with consistent observations.

Fluorescent Spectroscopy Revealed the Pulsatile Refolding by Adding Refold Buffer into Protein as Better Method of Renaturation of TT–KK–ZP3 Protein

To optimize the refolding process, solubilized recombinant TT–KK–ZP3 was refolded by the two protocols as described in Materials and Methods. Analysis by SDS-PAGE revealed that the protein renatured by adding buffer to the recombinant protein has slightly higher mobility (Fig. 4a, lane 1). This could have arisen due to the subtle differences in folded structures assumed by the recombinant protein during two different dilution methods which gave rise to "gel shift" [31]. Fluorescence spectroscopy studies of the recombinant protein refolded by the two
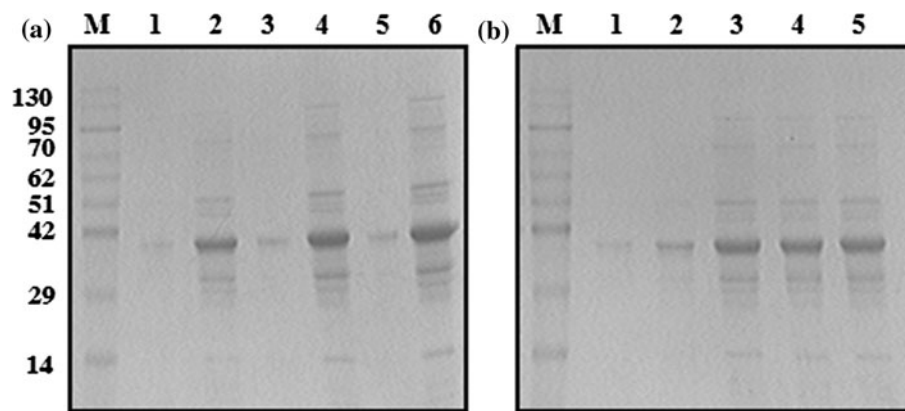
Fig. 2 Optimization of expression of the recombinant TT–KK–ZP3 protein. **a** Shows Western blot of *E. coli* M15 (pREP4) cells harboring TT–KK–ZP3 in pQE-60 expression vector induced with 1 mM IPTG for different periods of time (2, 4, 6 h) at 37 °C; *lanes 1, 3, 5* represent uninduced cells and *lanes 2, 4, 6* represent cells induced with IPTG for 2, 4, and 6 h, respectively. **b** Shows Western blot

profile of *E. coli* M15 (pREP4) cells harboring TT–KK–ZP3 in pQE-60 vector induced with different concentrations of IPTG (0.1, 0.3, 0.5, and 1.0 mM) for a constant time period (4 h) at 37 °C; *lane 1* represents uninduced cells and *lanes 2, 3, 4, 5* represent cells induced with 0.1, 0.3, 0.5, and 1 mM IPTG for 4 h, respectively. *Lane M* represents molecular weight markers
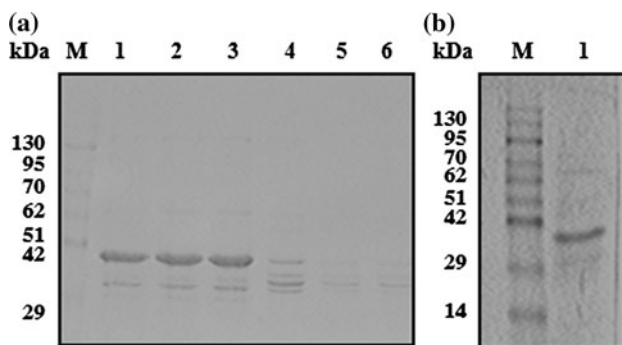

Fig. 3 SDS-PAGE analysis of the purification and solubilization of TT–KK–ZP3 IBs. **a** Shows Coomassie stained SDS-PAGE profile for solubilization of IBs under different experimental conditions; *lanes 1, 2,* and *3* represent soluble fractions of the purified TT–KK–ZP3 when purified IBs pellet was solubilized with 100 mM citrate buffer, pH 4.0 containing 8 M urea only, 8 M urea with 5 mM β-mercaptoethanol, and 8 M urea with 10 mM β-mercaptoethanol, respectively, whereas *lanes 4, 5,* and *6* represent their insoluble counterparts. **b** Shows Coomassie stained SDS-PAGE profile of the purified and solubilized IBs; *lane 1* represents soluble fraction of purified IBs washed with 50 mM Tris containing 5 mM EDTA, 2 mM DTT, 2 % sodium deoxycholate, and 100 mM NaCl and solubilized in 100 mM citrate buffer, pH 4.0 containing 8 M urea and 10 mM β-mercaptoethanol. *Lane M* represents Molecular weight markers

protocols as well as denatured protein sample (as negative control) were performed as described in Materials and Methods. In this case, the denatured protein obtained by adding 6 M guanidine hydrochloride to renatured protein showed a significant amount of red shift in emission maxima as well as very less intensity of emission as compared to renatured protein (Fig. 4b). The data proves the presence of folded structure in both the renatured protein samples. Further analysis of emission spectra shows that Sample 1 which was obtained by adding buffer

into protein showed a slightly better folded structure as it showed emission maxima (265 A.U.) at wavelength (λ_{max}) of 339 nm, whereas the Sample 2, in which dilution during refolding was carried out by adding protein to the buffer, has shown emission maxima (245 A.U.) at wavelength 342 nm. Sample 2 showed not only a slight red shift but also lesser intensity of emission, signifying the presence of more folded structure in Sample 1. Further, when yields of both the samples of refolded protein were compared, it was found that Sample 1 had better yield ($\sim$6 mg protein/l of culture) as compared to Sample 2 ($\sim$4.5 mg/l culture). These observations support the fact that the better the folding, the less the aggregation and the higher the yield of protein. In both the cases, SDS-PAGE profile revealed that the purity of the recombinant TT–KK–ZP3 was $\sim$95 %.

CD Spectra of the Refolded Recombinant TT–KK–ZP3 Protein Revealed the Presence of Secondary Structure

Circular dichroism spectra of the refolded recombinant TT–KK–ZP3 showed peaks at 208 and 218 nm. As the α-helical proteins have negative bands at 222 and 208 nm and proteins with well-defined anti parallel β-pleated sheets (β-helices) have negative bands at 218 nm [6], CD spectrum studies of the refolded protein showed the presence of both α- and β-helices in its secondary structure. The denatured protein has lost this structural conformation (Fig. 5). The reason for the uneven spectra of the control sample below 210 nm may be due to the high concentration of guanidine hydrochloride (6 M) used to denature the protein. At such a concentration, guanidine hydrochloride absorbs too strongly to allow reliable CD data to be collected below 210 nm even using cells of short path length (0.02 cm). However, this is not a problem, if changes in the CD signals are used to assess the
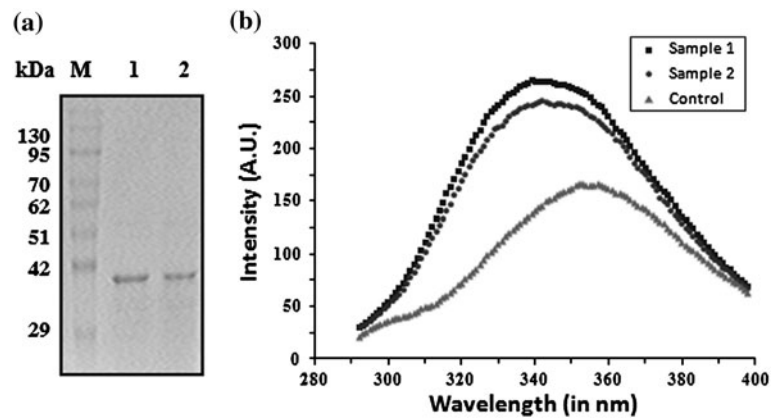
Fig. 4 Optimization of the renaturation of recombinant TT–KK–ZP3 protein. **a** Shows Coomassie stained SDS-PAGE profile of the purified recombinant TT–KK–ZP3 protein refolded either by gradual addition of 100 mM citrate buffer, pH 4.0 to the protein (*lane 1*) or by addition of solubilized protein to the citrate buffer (*lane 2*) and subsequently dialyzed against 20 mM Tris, pH 6.8. *Lane M* represents molecular weight markers. **b** Shows Fluorescence spectroscopy analysis of the renatured protein thus obtained using the above two protocols; *Sample 1*: Emission spectra of TT–KK–ZP3 refolded by adding buffer into the protein, *Sample 2*: Emission spectra of TT–KK–ZP3 refolded by adding protein into the buffer and control represents the same when Sample 1 was denatured by adding 6 M guanidine hydrochloride
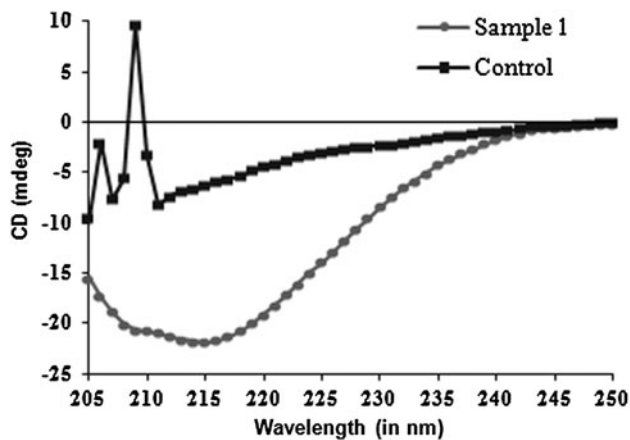

Fig. 5 CD spectral analysis of the renatured TT–KK–ZP3. The CD spectra of the renatured TT–KK–ZP3 protein, refolded by adding buffer into the protein (*Sample 1*) was recorded between 200 and 250 nm wavelengths using 300 μg/ml of the renatured protein. The CD spectra of the same recombinant protein denatured by adding 6 M guanidine hydrochloride (*control*) has also been shown

unfolding of a protein [13]. Both CD and fluorescence spectra validated the presence of a defined structure of the refolded fusion protein.

E. coli-Expressed Recombinant TT–KK–ZP3 Protein is Immunogenic in Female Mice and Antibodies Thus Generated Recognized Native ZP

Immunization of female FvB/J mice with TT–KK–ZP3 supplemented with PetGel A led to generation of antibodies against the recombinant protein (Table 1). Recombinant protein elicited predominantly Th-2-type of immune response as observed by high levels of IgG_1 isotype of antibodies as compared to IgG_{2a} isotypes (Fig. 6). Studies with PetGel A have shown that it is a safe adjuvant and can be used for vaccines meant for animal application [29]. Incorporation of T cell epitope of TT in recombinant dog ZP3 (TT–KK–ZP3) may lead to generation of higher antibody titers as it binds to various major histocompatibility

Table 1 Immunogenicity and contraceptive efficacy of the recombinant TT–KK–ZP3 protein in female FvB/J mice

Immunogen		Antibody titers in $AU^a \times 10^3$ (mean ± SEM)				Pups/litter (mean ± SEM)	% of animals failed to conceive	Pups/mated female
		Day 0	Day 20	Day 34	Day 49			
PetGel A	$n = 1$	<0.05[b]	<0.05[b]	<0.05[b]	<0.05[b]	0	10.0	7.1 ± 0.95
	$n = 9$	<0.05[b]	<0.05[b]	<0.05[b]	<0.05[b]	7.9 ± 0.59		
TT–KK–ZP3 + PetGel A	$n = 6$	<0.05[b]	0.85 ± 0.1	51.8 ± 6.7	77.7 ± 5.7	0	60.0	2.3 ± 1.05
	$n = 4$	<0.05[b]	0.58 ± 0.1	27.3 ± 3.9	47.7 ± 3.3	5.7 ± 1.31		

[a] AU represents antibody titers as reciprocal of the serum dilution giving an absorbance of 1.0 in ELISA

[b] Serum samples when tested at 1:50 dilution showed an absorbance of <1.0
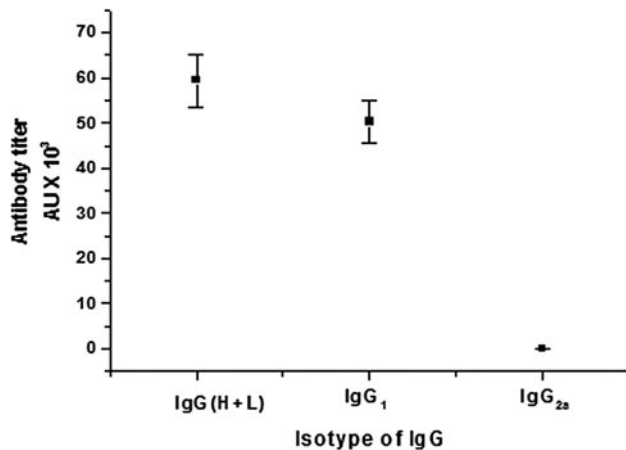
Fig. 6 Analysis of IgG$_1$ and IgG$_{2a}$ isotypes in mice immunized with *E. coli*-expressed recombinant TT–KK–ZP3. Female FvB/J mice were immunized with *E. coli*-expressed recombinant TT–KK–ZP3 as described in Materials and Methods. The immune serum samples corresponding to day 49 bleed were assessed for IgG$_1$ and IgG$_{2a}$ antibody titers against ZP3 in an ELISA. Plot represents the comparison between total IgG, IgG$_1$, and IgG$_{2a}$ antibody titers expressed as antibody units

complex (MHC) molecules and thereby likely to elicit immune response in larger proportion of the immunized outbred population. This will also avoid chemical conjugation of the recombinant dog ZP3 with "carrier" protein. Promiscuous T cell epitopes from a variety of antigens have great potential to provide T cell help that can be employed to generate humoral immune response against self proteins [10, 20].

The antibodies thus generated in female mice against the recombinant protein reacted with native ZP matrix surrounding the mouse oocytes in an indirect immunofluorescence assay, whereas a pre-immune serum sample from the same animal failed to show any reactivity (Fig. 7). It has been shown previously that antibodies generated against porcine ZP glycoproteins in male rabbits react with ZP isolated from various species including humans [32]. In the present study, we showed that antibodies generated against recombinant dog ZP3 reacted with native mouse ZP, which may be due to the fact that canine and mouse ZP3 at aa level exhibit an identity of 65 %.

Immunization with Recombinant TT–KK–ZP3 Led to Curtailment of Fertility

The above groups of immunized mice were mated with males of proven fertility to assess contraceptive efficacy of the recombinant TT–KK–ZP3. Only 4 out of 10 female mice ($p = 0.017$) immunized with TT–KK–ZP3 supplemented with PetGel A conceived subsequent to mating as compared to 9 out of 10 female mice receiving adjuvant alone (Table 1). A significant reduction ($p = 0.0027$) in the number of pups born/mated female in the group of animals immunized with recombinant TT–KK–ZP3 (2.3 ± 1.05) was also observed as compared to group receiving adjuvant alone (7.1 ± 0.95). Subfertility was also observed in animals immunized with recombinant TT–KK–ZP3 as compared to adjuvant alone (5.7 ± 1.31 pups/pregnant mice vs 7.9 ± 0.59 pups/pregnant mice; Table 1); which, however,

Fig. 7 Reactivity of polyclonal antibodies against recombinant TT–KK–ZP3 with mouse oocytes in an indirect immunofluorescence assay. Mouse oocytes ($n = 5$) were incubated with 1:20 dilution of either pre-immune or immune serum from FvB/J strain of female mice immunized with recombinant TT–KK–ZP3 and processed for their reactivity by an indirect immunofluorescence assay as described in Materials and Methods. Representative immunofluorescence profiles are shown. The *top panel* shows reactivity profile with pre-immune serum and *bottom panel* with immune serum. In both the panels, *left hand* sub-panel represents phase contrast and *right hand* panel represents immunofluorescence. The *scale bar* represents 50 μm
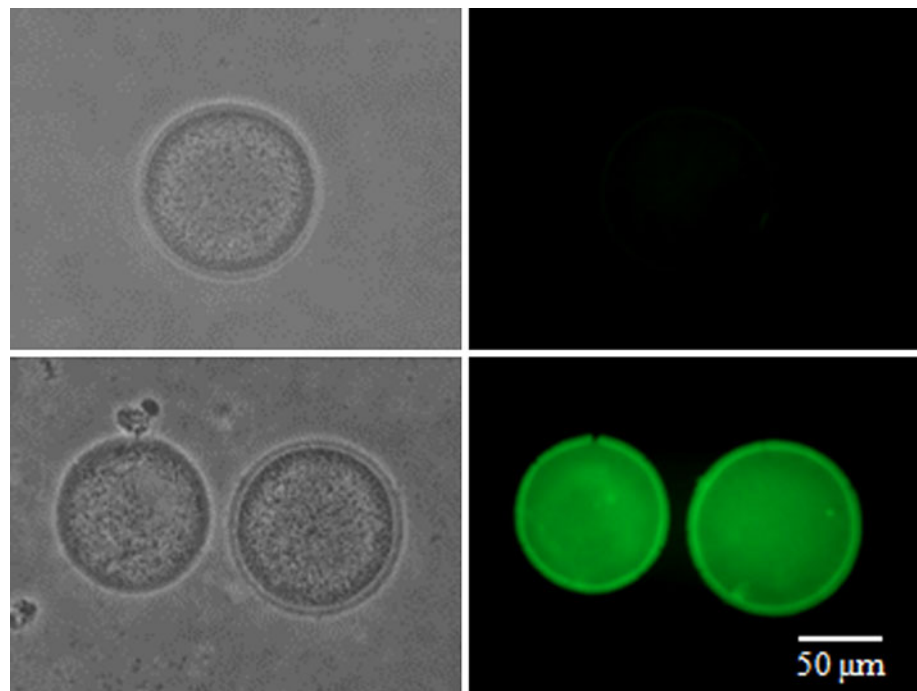
was not statistically significant ($p = 0.08$). Group of animals immunized with TT–KK–ZP3 that conceived subsequent to mating had lower mean antibody titers as compared to those which failed to conceive (Table 1). Although, a trend is observed that higher antibody titers are associated with infertility, studies comprising large number of animals need to be undertaken to determine the correlation between antibody titers and infertility. Further, from these pilot studies, it is difficult to arrive at protective antibody threshold that is critical to achieve sterility. The contraceptive effect of recombinant TT–KK–ZP3 is likely to be mediated by antibodies generated against ZP3 as TT component corresponds to promiscuous T non-B cell epitope of TT and is less likely to generate antibodies. In addition, active immunization studies in marmosets (*Callithrix jacchus*) with recombinant human ZP3 and its corresponding peptides showed that immunization with TT has no effect on fertility [30]. It is likely that these studies will facilitate the efforts to develop contraceptive vaccine for the management of street dogs populations, which is a major problem in several developing countries and thereby reduce the burden of rabies [25].

Conclusion

To conclude, the present study describes the development of a simple and effective protocol for purification and refolding of *E. coli*-expressed TT–KK–ZP3 without any affinity tag from IBs. CD and Fluorescence spectroscopy analysis revealed that the purified recombinant protein has been renatured and shows defined structural attributes. However, at this time it is not feasible to comment whether the folding is akin to native protein, as due to ethical and technical reasons, it is difficult to have highly pure dog ZP3 from ovaries to conduct similar studies. Nonetheless, the purified recombinant protein is immunogenic when delivered with adjuvants permitted for human and animal application and leads to curtailment in fertility. It will be interesting to carry out active immunization studies in female dogs with the recombinant TT–KK–ZP3 to investigate its potential as contraceptive vaccine for management of street dog populations.

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