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In silico study of conserved region from recombinant human ZP3 (rhZP3) produced in *E.coli* BL21 competent cells for developing woman immunocontraception

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Abstract: The aim of this study is to constract the hZP3 gene and to identify the hZP3 clone by using PCR and sequence analysis. Amino acid sequence will be used to identify conserve region of rec-hZP3 using in silico analysis. The sample were derived from human blood. Blood DNA was isolated by salting-out method and then amplified by PCR with a pair of specific primer. The PCR-product was cloned into vector pET-28a and the pET28a-hZP3 clone was transformed into *E. coli* BL21 competent cells. The pET28a-hZP3 was confirmed by PCR and DNA sequencing. The PCR product of pET28a-hZP3 clone was single band of 122 bp. These results indicated that the hZP3 gene inserted into pET28a properly. In silico analysis showed that amino acid conserved region SQWSRSASRNRR were homology among various species mammalia and have 3 conserved regions of CR 1 (PIECRYPRQGNVSS,137-150 aa), CR 2 (DVTVGPLIFL,359-368 aa) and CR 3 (SQWSRSASRNRR ,341-352 aa). The SQWSRSASRNRR sequence confirmed as an epitope recommended for development woman immunocontraception.
Keywords: Immunogenicity, Woman Immunocontraception, recombinant human ZP3.

Introduction

Intervention of fertility, via immunocontraception, has became interesting topic research for many years. The mechanism of intervention fertility agent for vaccine contraception is stimulated by imune reaction and led to block of fertility. Researches of immunocontraception have been conducted in several species, but have not yet been done in bovine. As well as known that hormonal contraception up to now is still having side effects[1].

In the previous studies, some successful applications of native protein of bovine zona pellucida (bZP) for contraceptive vaccine were reported in various species of wild animals. Native bZP3 consists of four major glycoprotein components: bZP1-bZP2 (110 kDa), bZP3 (80kDa) and bZP4 (54 kDa). bZP1 and bZP2 were migrated together so that result the same electrophoretic band. These were could be separated by two-dimensional electrophoresis[2]. Our previous study for active immunization with these components showed that bZP3 could be used for induction of a temporary infertility in several animals model[3]. Since glycoproteins are generally much more immunogenic than nonglycosylated proteins, production of recombinant ZP3 protein was

attempted in bacteria cell. Active immunization with recombinant non-human primate ZPB expressed in *E. coli* and coupled to DT as carrier protein was inhibited fertility in baboons[4]. These studies suggest that glycosylated recombinant protein would be a good candidate for the antigen of contraceptive vaccines.

Studies in several animal models have demonstrated that active immunization of female subjects with ZP leads to a block of fertility. Animals immunized with rec75 showed interference in the folliculogenesis, whereas animals immunized with rec55 had normal ovarian follicular development and hormonal profile (Paterson et al., 1998)5. Antibodies against rec55 also inhibited in vitro monkey sperm–egg interaction. Female marmosets (*Callithrix jacchus*) immunized with recombinant human (h) ZP3, expressed in CHO mammalian cells, showed high circulating antibody titres and long-term infertility. Rabbit polyclonal antibodies as well as murine monoklonal antibodies against r-bmZPB expressed in *E.coli* significantly inhibit, in vitro, the binding of human spermatozoa to antibody treated zona-encased human oocytes[4].

In the present study, we cloned human ZP3 from human blood then combined in plasmid pET-28a and expressed in *Escherichia coli* BL21. Subsequently, identication of the hZP3 clone was done by using PCR and sequence analysis. Amino acid sequence serve to identify conserve region of rec-hZP3 using in silico analysis.

Materials and Methods

Preparation of DNA from Female Human Blood

Blood DNA was isolated by salting-out method[6]. Blood from EDTA tube transfered to propilen tube and added RBC lysis. Then Incubated in room temperature for ten minutes and centrifuged at 3000 rpm ten minute in 25°C. Pellet was added with cell lysis solution, gently mix the solution, then incubated in 37 oC shaker waterbath. Added with EtOH absolute and centrifugated at 10.000 rpm and the pellet were mixed with 70% ethanol. After that, the pellet were dried and added with TE buffer pH 7.6 and stored in -20ºC. The quality and quantity of DNA were measured by using Nano Drop spectrophotometer and 1% agarose gel electrophoresis.

DNA Amplification

DNA from blood was amplified by primer designed specifically. Polymerase Chain Reaction (PCR) program: hot start 94ºC for 1 min, denaturation 94ºC for 30 sec, annealing 57ºC for 30 sec, and extension 72ºC for 45 sec (40 cycles), and then post extension 72ºC for 7 min. Oligonucleotide primers (5' to 3') hybridizing to the coding strand include: reverse 5'ATGC CTC GAG CTA CCT GCG GTT ACG GGA AGC-3' and forward 5'ATGC GGA TCC ATG GTG GAA GGC TCG GCT GAC-3' by 10 pmol/µl concentration, incorporating BamH1 and Xho1 restriction sites, respectively. PCR products were measured qualitatively by using 2% agarose gel electrophoresis. PCR products were sequenced by same primer to identified hZP3 gene.

Preparation of DNA vector

Plasmid pET-28a was transformed into *E.coli* strain BL21 competent cells (Novagen) and cultured the clone in Lennox Broth (LB) agar medium containing kanamycin (0,05 mg/ml) and chloramphenicol (0,034 mg/ml) antibiotics. DNA plasmid was isolated by miniprep method (Birnboim and Doly, 1979)7. The quality and quantity of DNA plasmids were measured by using Nano Drop spectrophotometer and 1% agarose gel electrophoresis.

Gene cloning of hZP3 gene into plasmid pET-28a

DNA plasmid and PCR product were digested by *BamHI* and *XhoI* enzymes. After purification by elution method, between DNA pET28a-digested and ZP3 amplified gene target were ligated by T4-DNA ligase. The pET28a-ZP3 construction transformed into *E. coli* BL21 competent cells and cultured on LB-selected agar medium containing kanamycin and chloramphenicol antibiotics for blue white colony screening. Schematic of DNA pET-28a and ZP3 gene recombinant was constructed on Figure 1.

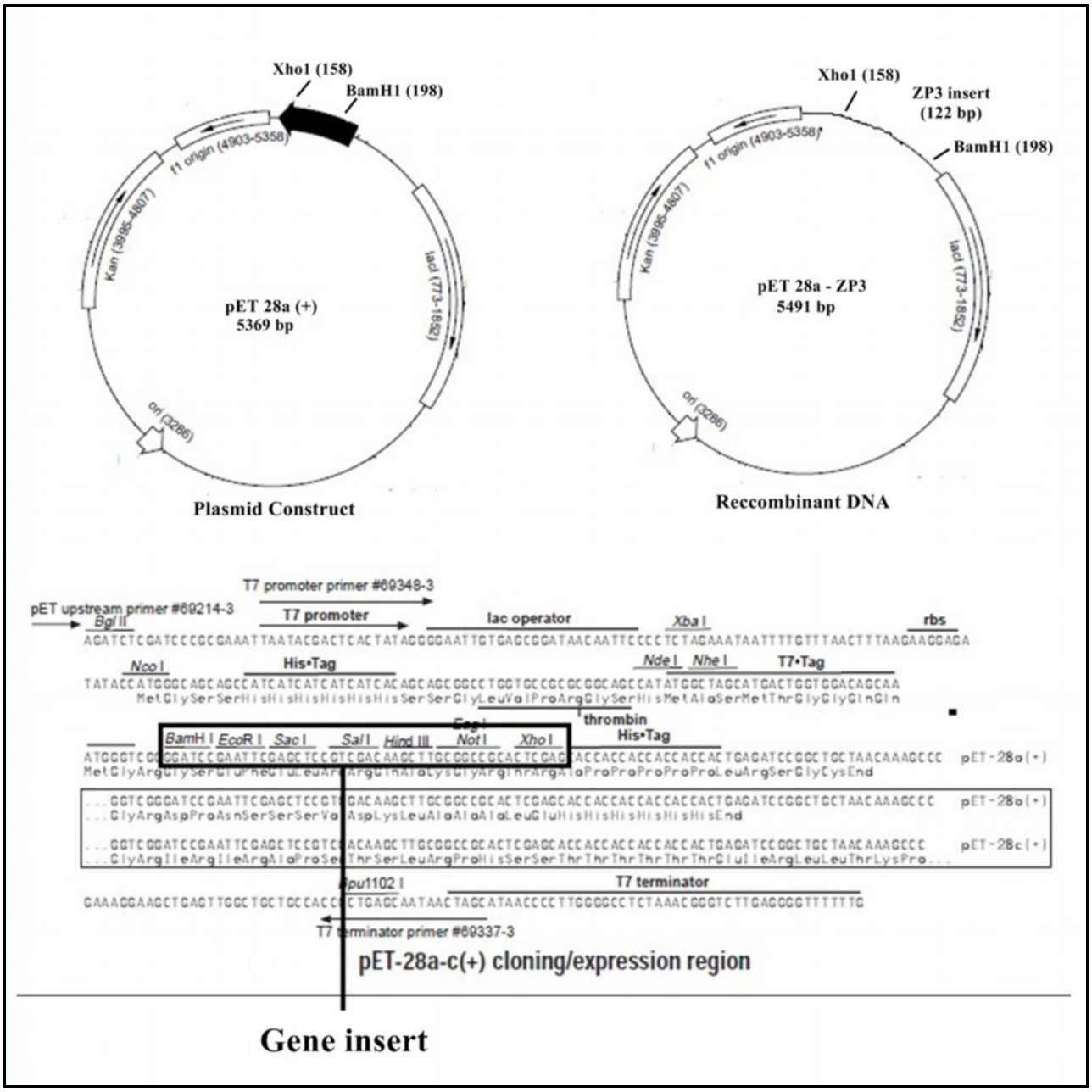

Figure 1.The Construction of DNA pET-28a and ZP3 gene Recombinant

Confirmation of the recombinant DNA by PCR

To confirm the recombinant DNA, white colonies were isolated by miniprep method. PCR analysis is using a pairs from same ZP3 primer. PCR products were measured qualitatively by using 2% agarose gel electrophoresis.

In Silico Analysis

PCR products were purified first and alignment of PCR product of hZP3 before cloning and after cloning using Bioedit. Conserved region of ZP3 between hZP3 PCR product with NCBI were identified via BLAST analysis, available at the Conserved Domain Database at NCBI.

Results

The Cloning of Human Zona Pellucida 3 Gene in *E. Coli* BL21

The 122 bp sequence at the time before cloning, which showed up to 100% homology with the published ZP3 sequence, was amplified from pET 28a vector at *Xho1* and *BamHI* sites (Figure 2A and 2C). Authenticity of the insert in the clone was confirmed by PCR (Figure 2B) and sequencing (Fig. 3). The isolation of DNA resulted in which DNA concentration of human namely 5.9 µg/ml. Target gene of human Zona Pellucida 3 (ZP3) was 122 bp (Figure 2A). The 122 bp bands of ZP3 was observed on female human blood. The result of alignment of sequencing product showed that gene sequence from gene bank have similarity with ZP3 recombinant sequence from human (Figure 2C). Three white clones for human ZP3 were identification of recombinant DNA with PCR amplification using a pair of bZP3-F-BamH1-Exon 8-944 and bZP3-R-Xho1-Exon 8-1066 primer was shown in figure 2B. We detected single band of 122 bp to human ZP3 recombinant.

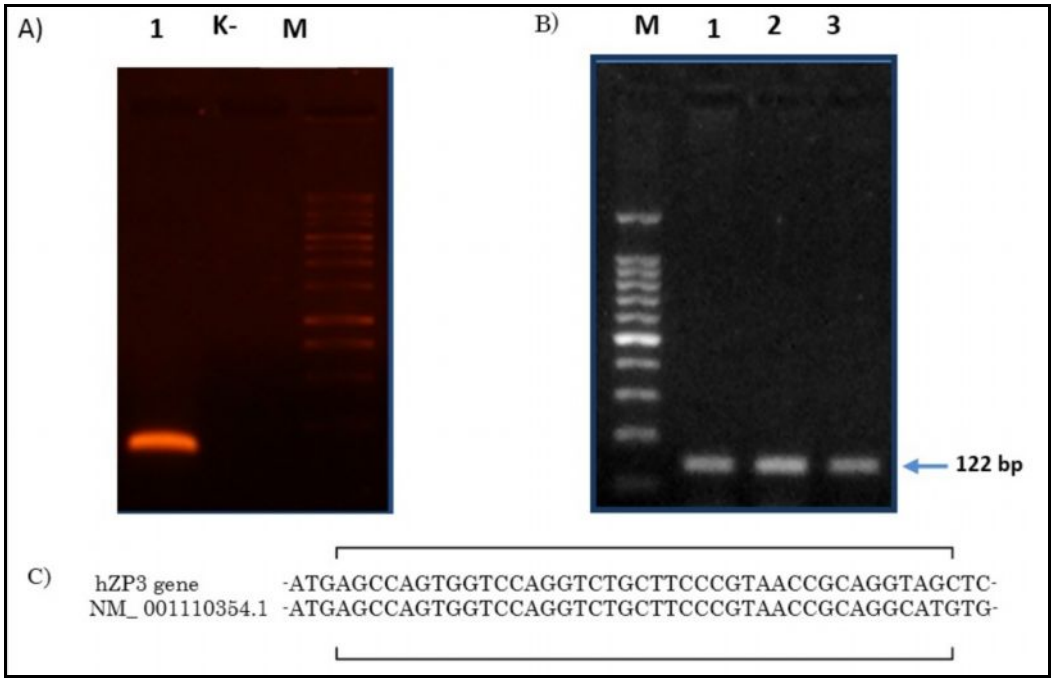

Figure 2. DNA target and PCR product of hZP3 before cloning (A) and after cloning (B) identified by specific ZP3 primer. Alignment of hZP3 from gene bank and sequencing product 9C); M: Marker; K- : Negative control; lane 1-3: Human ZP3

In Silico Analysis

The sequences of recombinat hZP3 were analyzed by BLAST for their similarity with H. sapiens sequences in the GenBank. The product rec. hZP3 have similarity amino acid sequence that already available in the GenBank with amino acid sequence of SQWSRSASRNRR (Figure 3). Based on BLAST analysis, the amino acide sequence of ZP3 have three regions, namely CR 1 for PIECRYPRQGNVSS (137-150 aa), CR 2 for DVTVGPLIFL (359-368 aa) and CR 3 for SQWSRSASRNRR (341-352 aa) (Table 1). BLAST analysis showed that SQWSRSASRNRR has similarity sequence with human, horses and hamsters for 100%, 82% and 75 %, respectively.

The analysis showed that conserved region of ZP3 has known to have three regions, namely CR 1 for PIECRYPRQGNVSS (137-150 aa), CR 2 for DVTVGPLIFL (359-368 aa) and CR 3 for SQWSRSASRNRR (341-352 aa) (Table 1). Human ZP3 sequence have glycans site (Asn-X-Thr/Ser) (Table 2). Amino acid region SQWSRSASRNRR were homology among various species mammalia.

Table 1. Conserved region of ZP3

No	Total sequence of amino acid	CR1	CR2	CR3
1	Human (424 aa)	137-150 piecryprqgnvss	359-368 dvtvgplifl	341-352 sqwsrsasrnrr
2	Horse (*Equus caballus*) 426 aa	137-150 piecryprqgnvss	359-368 dvtvgplifl	341-352 rqwhksasrnrr
3	Hamster (*Cricetulus griseus*) 403 aa	117-130 piecryprqgnvss	339-348 dvtvgplifl	321-332 srwpksasrnrr

Table 2. Glycan Site of Conserved Region

	CR1	CR2	CR3
Amino acid	**piecryprqgnvss**	**Dvtvgplifl**	**Sqwsrsasrnrr**
	Pro-Ile-Glu-Cys-Arg-Tyr-Pro-Arg-Gln-Gly-Asn-Val-Ser-Ser	Asp-Val-Thr-Val-Gly-Pro-Leu-Ile-Phe-Leu	Ser-Gln-Trp-Ser-Arg-Ser-Ala-Ser-Arg-Asn-Arg-Arg

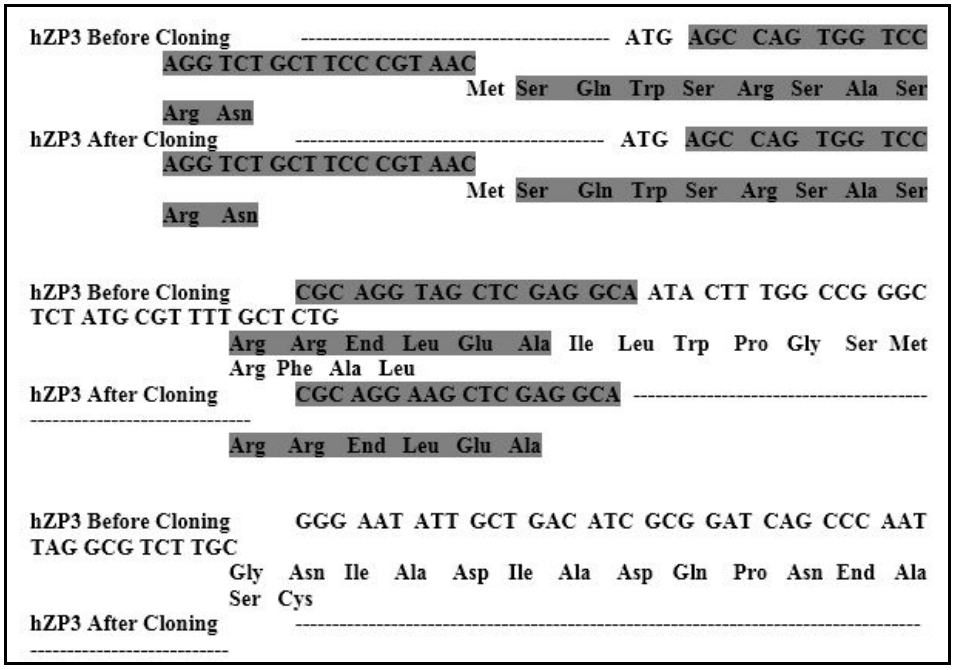

Figure 3. Sequence DNA-amino acid between human ZP3 and bovine ZP3 before and after cloning

Discussion

The PCR products of hZP3 clones indicated that a pairs of bZP3-F-BamH1-Exon8-944 and bZP3-R-Xho1-Exon8-1066 primers recognized target ZP3 gene specifically. This may indicate that hZP3 gene was inserted into plasmid pET 28a properly. This study uses *E.coli* BL 21 as competent cells. *E.coli* BL 21 have high stability in protein expression. Production of recombinant protein using bacteria system has been studied and showed that efficacy of active immunization with recombinant bonnet monkey ZP3 expressed in *E.coli* and added carrier protein to inhibit fertility and not inhibit ovarian function in babons[4].

In the present studies, the conserved region of the ZP3 protein exhibited potential as contraceptive. This region may contribute towards the species-specificity of sperm-egg binding. The ZP glycoproteins are highly glycosylated and have both N- and O-linked glycans[8]. Both specific sugar residue as well as peptide moieties

have been recommended as mediators of sperm-egg recognition, attachment and binding and acrosomal exocytosis in various mammalian species including human and bovine.

Structural study of the N-linked carbohidrate chains of ZP from bovine showed that a high mannose type neutral oligosaccharide was present in the bovine zona proteins. α-Galactose, which is present in the mouse zona proteins but absent from porcine zona proteins, was found in the core region of a triantennary acidic bovine zona protein. The biological functions of α-galactose and α-mannose residues at the non-reducing has been reported that mouse sperm binds to α-galactose and rat sperm binds to α-mannose in the glycoprotein of the homologous egg system[9].

Conclusion

The PCR product of pET28a-hZP3 clone which was single band of 122 bp. hZP3 gene was inserted into plasmid pET 28a properly. There are similarities between the amino acid sequences of human before and after cloning including SQWSRSASRNRR which have 12 amino acid from position 341-352 aa. These sequences have glycans site (Asn-X-Thr/Ser). This region may recommended towards the species-specificity of sperm-egg binding. Amino acid conserved region SQWSRSASRNRR were homology among various species mammalia.

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