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For the development of a ZP based contraceptive vaccine, using a homologous r-ZP3 antigen, the bonnet monkey, closely related to humans in evolution, has been used as a model system.

The cDNA encoding bZP3 was amplified by PCR, cloned and sequenced. Analysis of the sequence revealed that bZP3 cDNA had a single ORF of 1272 nt encoding a polypeptide of 424 aa residues, with a calculated molecular mass of 47.04 kDa. Comparison of the bZP3 with other reported sequences revealed that it had the highest reported identity of 93.9% with the hZP3. The hydrophobicity profiles of the bZP3 and hZP3 showed a close overlap. Two major hydrophobic domains at the N- and the C-termini of the bZP3 protein correspond to the signal sequence and the transmembrane-like domain, which would be cleaved in the mature extracellular bZP3 protein. The domain (aa 318-348), which is relatively poorly conserved among most species, and has been implicated as a sperm receptor site in the mouse model, is well conserved between the bZP3 and hZP3.

Attempts to express the full length bZP3 protein in E. coli were not successful. Subsequently, an internal fragment, excluding the N-terminal signal sequence and the C-terminal transmembrane-like domain of the bZP3, was amplified by PCR and cloned in the pQE30 vector. r-bZP3 was expressed with a N-terminal His6 tag, to aid its convenient purification, in E. coli strains SG13009[pREP4] and M15[pREP4]. Immunoblots using a murine MAb, MA-451 (raised against pZP3β, and crossreactive with bZP3) revealed a predominant band of 45-50 kDa besides lower molecular weight fragments, which could be due to degradation or premature termination of the protein during translation. r-bZP3 was expressed in protease mutant strains, BL21(DE3) and BL21(pLysS), lacking the ompT and the lon proteases, and in the DF5 strain, which carries a mutation in the pir gene. r-bZP3 expressed in BL21(DE3) and DF5 strains did not show the presence of the lower molecular weight bands. However, since mutants for different proteases showed the same effect, it was not possible to attribute the degradation to a specific protease. Antisera generated in monkeys against synthetic
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peptides from the N-(23-45 aa residues) and C-(300-322 and 324-347 aa residues) termini of the precursor bZP3 sequence reacted with the r-bZP3 protein in ELISA. The conditions to maximize expression of r-bZP3 were optimized. In batch shake flask cultures, 10 mg/L of r-bZP3 was purified from SG13009[pREP4] cells and 7.5 mg/L from BL21(DE3) cells.

The ZP proteins are extensively glycosylated in nature and the OS residues have been implicated as having a vital role in the sperm receptor function of ZP3. In order to obtain high level expression of a glycosylated r-bZP3, the gene encoding bZP3 was cloned in the BEVS under the polyhedrin gene promoter for expression in the Sf9 insect cell line. The full-length r-bZP3 (aa 1-424) expressed by cells infected with virus construct V1, was not secreted and was recognized as a 50-60 kDa doublet in Western blots using MA-451. The authenticity of expressed proteins was verified using Abs against N- (aa 23-45) and C- (aa 300-322) terminal peptides corresponding to the bZP3 sequence. Modified constructs were designed to evaluate the importance of major and minor hydrophobic domains within bZP3 on expression and secretion. Expression of bZP3 (aa 1-348) lacking the C-terminal major hydrophobic domain using recombinant virus construct V2, did not lead to its secretion from infected Sf9 cells but resulted in a 40-50 fold increase in bZP3 expression levels as seen by 35S-methionine incorporation studies. Importance of the minor hydrophobic regions in expression and secretion was further evaluated by using bZP3 constructs expressing aa 23-76 (virus construct V3) and aa 23-348 (virus construct V4) inframe with an insect derived secretory signal (gp67) fused to GST. The longer bZP3 stretch in V4 inhibited secretion of gp67-GST-bZP3 fusion protein and also caused reduction in expression levels to one-third as compared to V3. Indirect immunofluorescence localization studies revealed that deletion of the transmembrane domain also changed the distribution pattern of bZP3 in infected Sf9 cells. These results demonstrated that hydrophobic domains within the bZP3 protein play a vital role in determining the efficiency of expression and secretion in the baculovirus expression system.
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The r-bZP3 expressed in SG13009[pREP4] was purified on Ni-NTA resin under denaturing conditions and conjugated to DT, and that expressed in BL21(DE3) was conjugated to DT and TT. Immunization of a female rabbit with r-bZP3-DT conjugate generated Abs reactive with r-bZP3 in ELISA. Rabbit anti-r-bZP3 Abs reacted with pZP3β, a homolog of bZP3 in immunoblots, but did not react with pZP3α, thereby showing specificity to the ZP3 family of glycoproteins. Anti-r-bZP3 Abs also reacted with the native bonnet monkey as well as human ZP.

Active immunization studies were carried out in female bonnet monkeys showing normal cyclicity. Five animals were immunized with the r-bZP3 (expressed in SG13009[pREP4])-DT conjugate and boosted periodically using adjuvants permissible for human use. Four of the 5 animals generated a moderate anti-r-bZP3 Ab response and a high anti-DT response as a result of the immunization and were put for continuous mating with males of proven fertility. Two of the four animals in this group became pregnant. In order to eliminate a bias in the antigen preparation towards truncated molecules lacking the C-terminal region of the bZP3 protein, a second group of 3 monkeys was immunized with the full length r-bZP3 expressed in the BL21(DE3) cells. Both r-bZP3-DT and -TT conjugates were used together in the primary injection and boosters were administered alternately with either r-bZP3-DT or -TT conjugates, to reduce the high anti-carrier Ab response that was observed in the first group. Though anti-carrier response in this group was considerably reduced, a higher anti-r-bZP3 response was not elicited. In this group 2 of the 3 monkeys became pregnant despite moderate anti-r-bZP3 Ab titres. Failure to protect the animals immunized with r-bZP3-DT/TT along with adjuvants permissible for human use against conception has been discussed in light of thresholds of the protective Ab titres and Ab repertoire. These studies will further help in the careful design of effective immunocontraceptive vaccines based on ZP glycoproteins.