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(54) **Title:** ALGINATE CHITOSAN NANOFORMULATION OF OMPA - A SHIGELLA PROTEIN SUBUNIT

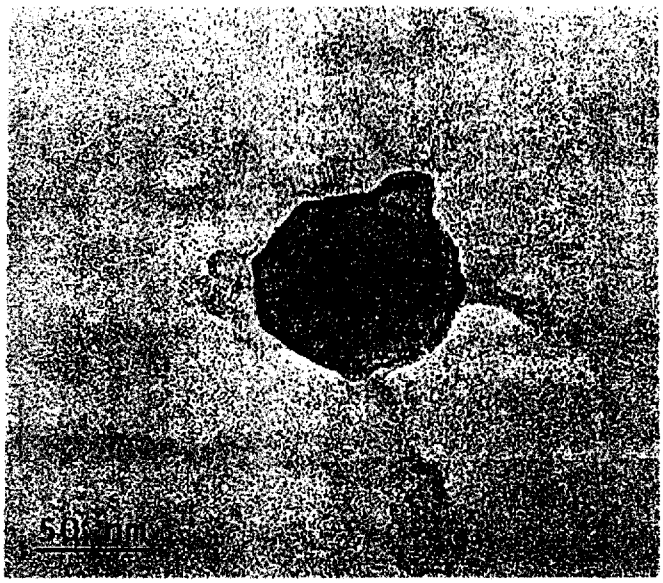

Figure : 1

(57) **Abstract:** A Formulation of OmpA comprising essentially of: a) OmpA protein as active molecule obtained as a product of OmpA gene inserted in a plasmid comprising a novel set of forward and reverse primer set, and b) Alginate chitosan nanoparticles as vehicle.

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FIELD OF THE INVENTION:

This invention relates to a formulation for immunogen improved delivery system and a process for the preparation thereof.

This invention further relates to a novel immunogen delivery system comprising an alginate chitosan nanoformulation of OmpA, a *Shigella* protein subunit vaccine against shigellosis.

BACKGROUND OF THE INVENTION;

Shigellosis is one of the major diarrheal diseases leading to high morbidity levels among the elderly and infant populations. But there is no potent vaccine available to prevent. Scientists and academician are trying to prepare a vaccine against shigellosis.

Endogenous peptides, protein, and oligonucleotides are among the main drugs which attract much attention because of their great potentials in treating chronic diseases. However, the extreme in vivo environment of human body has always limited the therapeutic applications of these substances. Polymeric nanoparticles have attracted much attention as delivery systems due to their ability in overcoming the physiological barriers and protecting and targeting the loaded substances to specific cells. Naturally occurring polymers such as chitosan (CS) have been studied to form nanoparticles. CS is a biodegradable polysaccharide, and it is derived from deacetylation of chitin. Apart from its biocompatibility, the low toxicity, hemostatic, and bacteriostatic properties also contribute to its various applications in pharmaceutical field. Several anions have been investigated to crosslink CS like sodium sulphate and dextran sulphate (DS). DS is able to modify protein and siRNA entrapment efficiency (EE) without the use of hardening agents and control the rate of drug release due to its high charge density. Besides DS is a cheap material, it produces mechanically more stable nanoparticles compared to the pentasodium tripolyphosphate (TPP).

Chitosan nanoparticles have been used in the art as a novel drug delivery system. The chitosan nanoparticles, because of their biodegradability, biocompatibility, better stability, low toxicity, simple and mild preparation methods, offer a valuable tool to novel drug delivery systems in the present scenario. Besides ionotropic gelation method, other methods such as microemulsion method, emulsification solvent diffusion method, polyelectrolyte complex method, emulsification cross-linking method, complex coacervation method and solvent evaporation method are also in use. The chitosan nanoparticles have also been reported to have key applications in parentral drug delivery, per-oral administration of drugs, in non-viral gene delivery, in vaccine delivery, in ocular drug delivery, in electrodeposition, in brain targeting drug delivery, in stability improvement, in mucosal drug delivery in controlled drug delivery of drugs, in tissue engineering and in the effective delivery of insulin. The publication 'Chitosan nanoparticles: a promising system in novel drug delivery', to Nagpal K[1]. Singh SK, Mishra DN., and Chem Pharm Bull (Tokyo). 2010 Nov; 58(1 1): 1423-30, discloses the origin and properties of chitosan and its nanoparticles along with the different methods of its preparation and the various areas of novel drug delivery where it has got its application.

The article Chitosan nanoparticles as a drug delivery system to A Krishna Sailaja, P. Amareshwar and P. Chakrabarty (Research Journal of Pharmaceutical, Biological and Chemical Sciences) is a review article which summarises the available preparation techniques and the various applications of Chitosan. The article 'Development of Chitosan nanoparticles as a stable drug delivery system for protein/ siRNA, to Haliza Katas et al (International Journal of Bio-materials, vol. 2013 (2013) Article ID 146320', discloses the modulation of preparative parameters on the physical characteristics and colloidal stability of CS NPs. CS NPs were fabricated by ionic interaction with dextran sulphate (DS) prior to determination of their

storage stability. The smallest CS NPs of nm with a surface charge of mV were produced when CS and DS were mixed at pH 4 and with a DS: CS mass ratio of 0.5: 1. An entrapment efficiency of 98% was achieved when BSA/siRNA was loaded into the nanoparticles.

The article 'Chitosan Nanoparticle - A Drug Delivery System', to Ashish Kumar Kosta, T.MohitSolakhia, Dr. Shikha Agrawal International Journal of Pharmaceutical & Biological Archives 2012; 3(4):737-743 discloses that Chitosan Nanoparticles are good drug carriers because of their good biocompatibility and biodegradability, and can be readily modified. As a new drug delivery system, they have attracted increasing attention for their wide applications. This paper reviews was published on Chitosan Nanoparticles including its preparation methods, modification, and applications.

However, nothing in the prior art discloses a formulation for delivery of immunogen OmpA, which can protect the protein from the acid barrier in the stomach and increase the bioavailability of the protein.

Hence, there is a need in the art to provide a formulation for delivery of the immunogen, which gives higher immunogenicity and protective efficacy than OmpA protein used alone.

OBJECTS OF THE INVENTION;

It is therefore the principal object of the present invention is to design a vaccine comprising OmpA nanoformulations as active molecule vehicle upon chitosan nanoparticles.

Another object of the invention is to provide a formulation for immunogen delivery system helping to protect the immunogen from the acid barrier of the stomach.

Yet another object of the invention is to provide a formulation for immunogen delivery system, which increases the bioavailability, biodegradability, biocompatibility of the immunogen.

Still another object of the invention is to provide a formulation for immunogen delivery system, which gives higher immunogenicity and protective efficacy over OmpA protein used alone with better stability and low toxicity.

Further another object of the present invention is to design novel primer according to the sequences of ompA gene.

Still further object of the present invention is to design a readily reproducible plasmid comprising ompA gene along with the novel primers.

SUMMARY OF THE INVENTION:

A novel formulation for improved immunogen delivery system comprises substantially effective amount of alginate chitosan nanoparticles with OmpA protein of *Shigella* species. Alginate chitosan nano formulations of OmpA comprises essentially of OmpA protein as active molecule obtained as a product of ompA gene inserted in a plasmid comprising a novel set of forward and reverse primer set having novel sequences of

5'-AAAAAGACACATATGCG ATT GCAG-3'

5'- GTATCTCGAGAGCTTGCGCTGAGTTAC-3'

and alginate chitosan nanoparticles as vehicle.

A method of preparation of alginate chitosan nano formulation, said method comprising the steps of designing a plasmid comprising novel ompA forward and reverse primer set as insert wherein the underlined bases in the forward primer represents restriction site specific for Ncol and the underlined bases in the reverse primer represents restriction site specific for Xhol; transferring said recombinant plasmid to E. coli BL21 (DE3) for multiplication; isolation of

OmpA protein as a product from the E. coli BL21 (DE3) in the mid log phase of said bacteria wherein the transposable genetic material comprising essentially of said recombinant plasmid insert containing ompA forward and reverse primer set as insert forward and reverse primer set as insert; preparation of chitosan nanoparticles; and loading of OmpA protein to said chitosan nanoparticles.

The preparation of chitosan nanoparticles comprising the steps of preparation of chitosan solution by dissolving chitosan in 1% (v/v) acetic acid solution at concentration of 2mg/ ml; preparation of anionic tripolyphosphate (TPP) solution by dissolving TPP in distilled water at the concentration of lmg/ ml; addition of TPP solution into chitosan solution dropwise at 1:2 ratio; formation of chitosan colloid nanoparticles under mild agitation in room temperature condition; formulation of colloid chitosan nanoparticles at room temperature which was centrifuged at 35,000 rpm for 1 hr.; discarding of supernatant and re-dispersing of the deposit in distilled water; and final collection of the alginate coated chitosan nanoparticles.

The method of loading of OmpA protein to said chitosan nanoparticles comprises the steps of re-dispersion of colloid said chitosan nanoparticles in 25 ml of distilled water at concentration of 2mg/ mL under continuous ultra-sonication to disaggregate the nanoparticles; incubating OmpA protein obtained as a product from the E. coli BL21 (DE3) with said nanoparticles under mild agitation at room temperature condition for 15 minutes to make final concentration of OmpA protein 1 mg/ mL; loading of OmpA protein subunit to the chitosan nanoparticles by incubating OmpA with said nanoparticles under mild agitation for 15 minutes in room temperature condition; dropwise addition of OmpA loaded chitosan nanoparticles with pH 5.1 to the sodium alginate solution under mild agitation at pH 7 for 10-20 minutes; and final collection of the alginate coated chitosan nanoparticles into calcium chloride (CaC12) aqueous solution at concentration of 0.524

mmol/L to crosslink the alginate layer, presents on the surface of the nanoparticles.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Fig. 1 Illustrates TEM image of chitosan Nano particle.

Fig 2 and 2a illustrate TEM image of OmpA coated with chitosan and alginate.

Fig 3 Illustrates intensity distribution of A) Chitosan, B) Chitosan with alginate, C) OmpA loaded chitosan with alginate (CAOP), D) Alginate.

Fig 4 Illustrates Serum immunoglobulin titers in immunized sera were measured against OmpA protein of *Shigella sp.*

Fig 5 Illustrates agarose gel electrophoresis M- Marker, l-pET28a plasmid, 2-pET28a plasmid with OmpA gene.

Fig 6 Illustrates western blotting with anti his-tagged antibody. M-Protein pre-stained molecular weight marker, 1-BL21 *E.coli* containing OmpA without IPTG, 2-BL21 *E.coli* containing OmpA induced by IPTG

Fig 7 Illustrates SDS-PAGE of OmpA. M-Marker, 1-Whole cell lysate of BL21 *E.coli* containing OmpA without IPTG , 2-Whole cell lysate of BL21 *E.coli* containing OmpA induced by IPTG, 3-In Supernatant, 4-In pellet as inclusion body, 5-In Wash buffer no protein, 6-purified fraction of OmpA in Elution buffer

Fig 8 Illustrates western blot analysis of recombinant OmpA of S. *flexneri* 2a with antisera from CAOP immunized mice with the whole-cell lysate of *Shigella* lane 1. S. *dysenteriael* Astx NT4907, lane 2. S. *boydii* type 4 BCH612, lane 3. S. *sonnei* IDH00968, lane 4. S. *flexneri* 2a 2457T, lane 5. S. *flexneri* 3a C519, and lane 6. S. *flexneri* 6 C347.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS:

This according to the invention is provided a formulation for immunogen delivery system and a process for the preparation thereof.

In accordance with this invention, a novel formulation for immunogen delivery method has been developed by using chitosan alginate nano formulation with OmpA protein (CAOP) of *Shigella* sp. and the protective efficacy and immunogenicity of CAOP against homologous as well as heterologous *Shigella* strains in animal model has been studied. Also the duration of protection offered by CAOP has been studied.

Sodium-alginate and chitosan both are extensively used in encapsulation of drug for the purpose of sustained release. These are polysaccharide polymers, formed of repeating units joined together by glycosidic bond. Both polymers have the properties of an ideal carrier for drug delivery, such as biocompatibility, biodegradability, non-toxicity, and low cost. Thus the formulation prepared from chitosan alginate nanoparticle will effectively deliver the OmpA in the gut epithelia. The controlled release of OmpA from the said nanoformulations could maintain steadier levels of this protein in bloodstream for longer durations.

Chitosan nanoparticles are prepared by the ionic gelation of Chitosan Solution with anionic tripolyphosphate (TPP). Briefly, chitosan was dissolved in 1% (v/v) acetic acid aqueous solution at concentration of 2 mg/ml. Then, TPP was dissolved in distilled water at the concentration of 1mg/ml. Subsequently, TPP solution was added drop wise into chitosan solution at 1:2 ratio. Chitosan colloid nano-particles formed spontaneously under mild agitation at room temperature. Two hours later, chitosan colloid nano particles was centrifuged at 35,000 rpm for 1 hr. Then, the supernatant was discarded and the deposit was re-dispersed in distilled water.

After the preparation of chitosan nanoparticle, it is loaded with OmpA.

The loading of OmpA procedure has been given below:

Colloid chitosan nanoparticles were then re-dispersed in 25 ml of distilled water at concentration of 2 mg/ml under continuous ultrasonication to disaggregate the chitosan nano particles. The loading procedure was performed by incubating OmpA with chitosan nano particles under mild agitation at room temperature for 15 min so that final concentration of OmpA protein lmg/ml.

Preparation of alginate coated chitosan nano particles:

OmpA loaded chitosan nanoparticles suspensions with pH value at 5.1 were added drop wisely into sodium alginate solution (pH = 7.2) at concentration of 1 mg/ml under mild agitation for 10 min. Then the suspension was centrifuged at 3,400 rpm for 5 min, and the supernatant was discarded.

Finally, alginate coated chitosan nano particles were re-dispersed into calcium chloride ($CaCl_2$) aqueous solution (pH = 7.0) at concentration of 0.524 mmol/L to crosslink the alginate layer presents on the surface of chitosan nano particles.

The final formulation of OmpA loaded chitosan alginate is clearly disclosed in accordance with Figure 2.

Figure 3 illustrates the intensity distribution of a) chitosan, b) chitosan with alginate, c) OmpA loaded chitosan with alginate (CAOP) d) Alginate.

The cloning of OmpA protein has been given below:

a) Sequence of OmpA gene:

The ompA gene was searched out from NCBI genome sequence of *Shigella flexnen* 2a 2457T. This sequence was used for designing of primers required for cloning.

The sequence was used for designing of primers required for cloning. The primers are designed from the upstream and downstream sequence of ompA gene using commercially available primer designing software. Two novel restriction sites were introduced in the primers.

The unique primer sequence is as below with underlined restriction digestion site. Forward primer contains same sequence while reverse contains the complementary sequence.

The primers are given as below:

bjPrimer designing:

The primers were designed by IDT software. They are as follows,

OmpA forward primer (Ncol):

5'-AAAAAGACACATATGCG ATT GCAG-3',

OmpA reverse primer (Xhol):

5'- GTATCTCGAGAGCTTGCGCTGAGTTAC-3'

Internal primers:

5'-ACC AGG TTA ACC CGT ATG TTG GCT TTG -3'

5'- TGT TGA GTA CGC GAT CAC TCC TGA AAT C -3'

5'- GTT CAA CTT CAA CAA AGC AAC CCT GAA AC -3'

5'- TCG GAC AGA CCC TGG TTG TAA G -3'

5'-AGC TGG AGC CGG AGC AAC TAC TGG -3'

5'- CTG AGC TTT GTA TGC ACC GTT TTC AA -3'

c) Insert preparation:

Template DNA was prepared from *Shigella flexnen* 2a 2457t. Genes were amplified by PCR. PCR amplified product was resolved by electrophoresis and analyzed.

d) Vector preparation:

Plasmid pET28a was isolated and digested with Ncol and Xhol restriction enzyme.

Template DNA was prepared from *Shigella flexneri* 2a 2457t. Genes were amplified by PCR using the primers for 30 cycles. PCR amplified product was resolved in 1% agarose gel by electrophoresis and analyzed using Gel-Doc (Bio-Rad). PCR products were purified and digested with Xhol. Now vector plasmid pET28a was isolated and digested with Ncol and Xhol restriction enzyme.

e) Ligation:

Vector and insert was used for ligation. Insert part contains two overhang with those two restriction enzyme site. Now ligation was performed. Vector and insert was mixed with a molar ratio of 1:3 for ligation. The ligated construct was transformed into *E. coli* BL21 (DE3). The positively cloned cells were selected by using ampicilin as a selective marker. The selected cells were confirmed further by colony PCR by using the forward and reverse primers. The colonies containing the recombinant plasmid was identified and confirmed by DNA sequencing.

Recombinant protein was purified by Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography. After induction, recombinant cells were lysed by lysis buffer (composition: Benzonase, PMSF, EDTA Free Protease inhibitor cocktail, Triton X 100, Lysozyme, Benzamidine, Glycerol, Tris-HCl pH 7.5, NaCl, β-marcaptoethanol), centrifuged at 7,000 g for 15 mins at 4°C. Now, pellet was collected and mixed with solubilization buffer (composition: Tris-

HCl pH 7.5, NaCl, β-marcaptoethanol, Urea 8 M, Imidazole). Ni-NTA resin with solubilization buffer was loaded on the column and finally the purified protein was eluted using elution buffer (Tris-HCl pH 7.5, NaCl, 3marcaptoethanol, Imidazole 500mM) after washing with wash buffer (Tris-HCl pH 7.5, NaCl.β marcaptoethanol, Imidazole 30Mm and 50mM). The affinity purified fraction was dialysed in the buffer 100 mM Tris-HCl (pH-7.5)-150mM NaCl. The expression and purity of the recombinant protein was confirmed by running on 10% SDS-PAGE.

The overexpression has been performed by the below method:

The recombinant BL21 (DE3) E. coli containing the OmpA gene was grown on 5 ml LB broth containing ampicillin and incubated overnight at 37°C. An aliquot of the overnight cell culture was added into another LB medium (containing 100 µg/ml ampicillin) and grown to mid log phase and incubated at 37°C with shaking (200 rpm). Once an optical density at 600 nm of the cultures reached 0.4-0.6 (mid log), cells were induced with 0.1 mM isopropyl thiogalactoside (IPTG) in one test tube while other was not induced. Next these two were subjected to SDS-PAGE followed by western blot with anti-His antibody which confirms overexpression of OmpA protein.

The sequence of OMPA gene of Shigella flexneri 2a 2457T has been given below:

1 atgaaaaagacagctatcgcgattgcagtggcactggctggtttcgctaccgtagcgcag

61 gccgctccgaaagataacacctggtacactggtgctaaactgggctggtcccagtaccat

121 gacactggttttattcctaacaatggtccgacccacgaaaaccaactgggtgcaggtgct

181 tttggtggttaccaggttaacccgtatgttggttttgaaatgggttacgactggttaggt

241 cgtatgccgtacaaaggcgacaacatcaacggcgcatacaaagctcagggcgttcagctg

301 accgctaaactgggttacccaatcactgacgatctggacatctacactcgtctgggtggt

361 atggtatggcgtgcagacaccaaggctaacgtacctggtggcgcatcctttaaagaccac

421 gacactggcgtttctccggttttcgcaggtggtgttgagtacgcgatcactcctgaaatc

481 gctacccgtctggaataccagtggaccaacaacatcggtgacgcaaacaccatcggtact

541 cgtccggacaacggtctgctgagcctgggtgtttcctaccgtttcggtcagggcgaagca

601 gctccggtagttgctccagctccggcaccggaagtacagaccaagcacttcactctgaag

661 tctgacgttctgttcaacttcaacaaagcaaccctgaaaccggaaggtcaggctgctctg

721 gatcagctgtacagccagctgagcaacctagatccgaaagacggttccgtagttgttctg

781 ggttacactgaccgcatcggttctgacgcttacaaccagggtctgtccgagcgccgtgct

841 cagtctgttgttgattacctgatctccaaaggtatcccggcagacaagatctccgcacgt

901 ggtatgggcgaatccaacccggttactggcaacacctgtgacaacgtgaaacagcgtgct

961 gcactgatcgactgcttggctccggatcgtcgcgtagagatcgaagttaaaggtatcaaa

1021 gacgttgtaactcagccgcaggcttaa

Various experiments have done for the novel formulation as claimed hereafter. The detailing of the experiments and data associated with those have been discussed below:

The morphological characteristics of nano particles were examined by transmission electron microscopy. The particle size distribution will detected by Dynamic light scattering. These measurements were run at least three times with independent particle batches.

Figure 3 depicts the intensity distribution curves for A) Chitosan, B) Chitosan with alginate C) OmpA loaded chitosan with alginate (CAOP) and D) alginates.

According to Figure 1, Figure 2 and Figure 2a, TEM images have shown the morphological properties and surface appearance of nano particles. The

chitosan nano particles have nearly spherical shape and size range of about 50-350 nm whereas size range of CAOP particles are 240-700 nm.

The respective average diameters, measured by Zetasizer, of chitosan nano particles and OmpA-loaded nano particles were approximately 240 nm and 535 nm. The PDI value of chitosan nanoparticles was 0.256 while that of OmpA-loaded chitosan nano particles was 0.199, thus indicating a narrow and favorable particle size distribution (PDI<0.5)(Table 1).

Morphological characterization, size and surface charge:

In present study the results obtained by Zetasizer revealed that the OmpA - loaded nano particles are larger than the chitosan-TPP ones, possibly due to the coating of alginate, high molecular weight and large size of the OmpA protein molecules.

The table 1 is provided which illustrates the size, PDI and zeta potential of different chitosan and alginate samples:

TABLE 1:

SAMPLE	MEAN PARTICLES SIZE (NM)	PDI	ZETA POTENTIAL
Chitosan	136.0nm	0.385	+32.70
Chitosan with alginate	249.2nm	0.260	-40.54
OMPA loaded chitosan with alginate	535.9nm	0.470	-20.78
Alginate	327.9nm	0.331	-45.47

The effectiveness of the novel formulation as claimed herein after is illustrated with foregoing example:

Swiss male and female mice, six to seven weeks old, were taken from animal resource department of NICED. Mice were caged separately and maintained at 25°C with 75% humidity and fed sterile food and water under the care of full time staff and in accordance with the rules of the institutional animal ethical committee (IAEC) (Apro/77/24/ 11/2O10, Reg. No. NICED/CPCSEA (AW) 215/2009-2015).

Female mice were immunized orally at days 0, 7, and 14 with 50 µg per 100 µï of purified OmpA coated with chitosan-alginate (CAOP) using the concentration. Fifteen minutes before the oral immunization; each mouse was anaesthetized by intramuscular injection of a mixture of ketamine (35 mg kg^{-1} body weight; Sterfil Laboratories Pvt. Ltd, India) and xylazine (5 mg kg^{-1} body weight, AstraZeneca Pharma India Ltd, India). CAOP was introduced directly into the stomach through a mouse feeding needle (Harvard Instrument, USA). The same volume of PBS was given by oral administration to the non-immunized group. All immunized and non -immunized group of mice were returned to their cages and given limited amounts of sterile food and water.

Infectious dose determination for challenge:

The infectious dose and the challenge dose of each strain were listed in table 2. The ID50 was considered to be the dose which ensured ~10^6 -10^7 bacteria per gram of intestine of the challenged neonates, after six hour incubation in 3 to 4 days old suckling mice.

TABLE 2 : ID50 and challenge dose of *Shigella* strains

STRAIN	INFECTIOUS DOSE$_{50}$ (ID_{50}) ($X10^8$ ML^1)	CHALLENGE DOSE ($X10^9$ ML^1)

S. *dysenteriae* 1 (NT4907)	1± 0.241	5 ± 0.356
S. *flexneri* 2a (2457T)	3± 0.151	4± 0.158
S. *sonnei* (IDHO0968)	1± 0.275	5± 0.141

Values are means ± SEM of three independent experiments.

Death or visible side effects due to toxicity (such as ruffled fur or lethargy or diarrhea or weight loss) did not occur in mice after three successive oral immunizations with 50µg of purified OmpA coated with chitosan-alginate(CAOP). A significant level of protection after both first and second challenge studies were achieved in newborn mice of immunized dams. Most of the suckling mice from non-immunized mother became sick and eventually died between 10 and 16 hr of incubation (Table 4). More or less same results were obtained after both challenge studies. Control mice from non -immunized groups showed higher intestinal colonization (~10^7 CFU/gm of intestine) leading to Shigellosis. Dead mice from immunized groups showed colonization ~10^5 CFU/gm of intestine which was 100 fold lower than the rate of intestinal colonization in control mice (~10^7 CFU/gm of intestine) and alive neonates showed even lesser intestinal colonization (~10^3 CFU/gm of intestine). CAOP conferred 100% protection against *S. flexnen* 2a after both challenge studies. Protective efficacies against S. *dysentenae* 1 and S. *sonnei,* (Table 3) were above 75%. The above results suggest CAOP immunization could confer 83-100% passive protection against shigellosis in neonatal mice model.

Protective Efficacy against shigellosis to the neonatal mice:

The aim of our study is to develop an oral subunit vaccine with *Shigella* protein OmpA coated with chitosan-alginate. We used female mice model to measure the protective efficacy and to study the immune responses that are elicited following disease or immunization. Two groups of mice (Immunized

and Control, weighing between 25 g) were selected for oral immunization with *Shigella* protein OmpA coated with chitosan -alginate. Each group contained 10 mice. The immunization experiment was done according to the method of Sack et al (1988) and the challenge experiment was done according to the method of Fernandez et al. (2003).

100% homologous protection was observed against virulent wild serotype while in average 75% was achieved in heterologous protection challenged by S. *dysenteriael* (NT4907) and S. sonnei (IDH00968). High reciprocal increase of serum lgG antibody titer was observed during the period of immunization.

Immunoblot data of whole cell lysate (WCL) also supported strong homologous protection as well as heterologous protection against CAOP immunization.

TABLE 3. First and second challenge study in suckling mice from immunized mother

CHALLENGED STRAIN	EXPERIMENTAL GROUP	NO.OF NEONATES IN EACH GROUP	%O OF SURVIVAL AFTER FIRST CHALLENGE	PROTECTIVE EFFICACY L%L AFTER FIRST CHALLENGE	% OF SURVIVAL AFTER SECOND CHALLENGE	PROTECTIVE EFFICACY A (%L AFTER SECOND CHALLENGE
S.dysenteriae 1 (NT4907)	Control	10	10 (1/10)	77.78	20(2/10)	77.78
	Immunized	10	80 (8/10)		90 (9/10)	
S. flexneri 2a (2457T)	Control	10	10 (1/10)	100	10 (1/10)	100
	Immunized	20	100 (20/20)		100 (10/10)	
S. sonnei (IDH00968)	Control	10	10 (1/10)	88.89	20 (2/10)	77.78
	Immunized	10	90 (9/10)		90 (9/10)	

[a]Protective efficacy was calculated as {[(percent deaths of non -immunized mice)-(percent deaths of immunized mice)] ÷ [percent deaths of non-immunized mice]} x 100.

M. ELISA:

Blood was collected at days O, 7, 14, 28, 35, 56, 72 and 120, after the first oral immunization. The collected blood was allowed to clot at room temperature (RT) for 30 min and serum was isolated by removing the blood clot with a sterile toothpick, followed by centrifugation (91 g, 10 min and 4°C). After adding sodium azide (final concentration 0.05%), the sera were stored at -80°C (Schild et al., 2008) until use. IgG (whole molecule) response in immunized and non-immunized sera were measured by ELISA, essentially following the method developed by Keren (1979). Disposable polystyrene (Nunc, Denmark) micro titer wells were coated with 10O µL of CAOP and incubated for 18 h at 4°C. Wells were washed three times with PBS (pH 7.4). Nonspecific binding sites were blocked by incubating the wells with 200 uL of 5% bovine serum albumin (BSA; Sigma Chemical) for 2 h at 37°C. The wells were washed three times with PBS-T (PBS with 0.5% Tween-20) and incubated with serially diluted serum samples at 37 °C for 1" h. Following washing, 100 µL HRP-conjugated goat anti-mouse immunoglobulin (Sigma Chemical) was added to each well and the plate was incubated at 37 °C. After washing with PBS, the substrate o-phenyl-D-amine (OPD) was added to each well. The reaction was stopped after 10 min by adding 100 µL of 2 N Sulphuric acids and the reading was taken at 492 ± 2 nm wave length using an ELISA reader. The experiments were repeated three times with the immunized and non-immunized serum, collected from individual mice, before, during and after the immunization period.

Figure 4 illustrates these test results clearly with the help of a graph.

Western blot analysis:

Whole cell lysates (WCL) of six virulent strain of *Shigella* sp, S. *dysentenaelAstx* NT4907, S. *boydii* type 4 BCH612, S. *sonnei* IDH00968, S. *flexneri* 2a 2457T, S. *flexnen* 3a C519, S. *ftexnen* 6 C347 were resolved by

SDS-PAGE followed by immunoblot using alkaline phosphatase conjugated lgG (whole molecule), according to the protocol described previously.

Immunoblot by anti-CAOP sera against WCL (F1G.9) demonstrated that CAOP antiserum cross reacted with the whole cell lysates of S. *dysentenae* lAstx NT4907, S. *boydii* type 4 BCH612, S. *sonnei* IDH00968, S. *flexnen* 2a 2457T. S. *flexnen* 3a C519, S. *flexnen* 6 C347. The recombinant protein also showed reactivity with mouse antisera produced by challenged with whole cell virulent S. *flexnen* 2a suggesting the strong immunogenicity of the recombinant OmpA. This also proved that the antigenicity of the protein is maintained. As chromosomally encoded OmpA protein is conserved among *Shigella* strains and *E. coli* strains it will give heterologous protection against not only *Shigella* species but also against pathogenic *E.coli* strains.

The novel formulation helps to protect the protein from acid barrier of stomach and chitosan being mucoadhesive can adhere with the mucus membrane and increase the bioavailability of the protein.

Hence, this innovative formulation provides substantially higher immunogenicity and protective efficacy than that of OmpA protein itself.

WE CLAIM:

1. A Formulation of OmpA comprising essentially of:

a) OmpA protein as active molecule obtained as a product of *OmpA* gene inserted in a plasmid comprising a novel set of forward and reverse primer set, and

b) Alginate chitosan nanoparticles as vehicle.

2. The formulation of OmpA as claimed in claim 1 wherein said plasmid contains said novel sequences of

5'-AAAAAGACACATATGCG ATT GCAG-3'

5'- GTATCTCGAGAGCTTGCGCTGAGTTAC -3'

3. A method of preparation of formulation of OmpA, said method comprising the steps of:

a) designing a plasmid comprising novel ompA forward and reverse primer set as insert:

5'-AAAAAGACACATATGCG ATT GCAG-3'

5'- GTATCTCGAGAGCTTGCGCTGAGTTAC -3'

wherein the underlined bases in the forward primer represents restriction site specific for Ncol and the underlined bases in the reverse primer represents restriction site specific for Xhol;

b) transferring said recombinant plasmid to *E. coli* BL21 (DE3) for multiplication;

c) isolation of OmpA protein as a product from the *E. coli* BL21 (DE3) comprising essentially of said recombinant plasmid insert

containing ompA forward and reverse primer set as insert forward and reverse primer set as insert;

d) preparation of chitosan nanoparticles; and

e) loading of OmpA protein to said chitosan nanoparticles.

4. The method as claimed in claim 3, wherein said designing of plasmid is performed by common restriction sites specific to restriction enzymes Ncol and Xhol.

5. The method as claimed in claim 3, wherein said designing of plasmid is performed through ligation of vector and said primer inserts by mixing vector and insert in a molar ratio of 1:3.

6. The method as claimed in claim 3, wherein said isolation of OmpA protein as a product from the *E. coli* BL21 (DE3) is performed in the mid log phase of said bacteria.

7. The method as claimed in claim 3, wherein said preparation of chitosan nanoparticles comprising the steps of:

a) preparation of chitosan solution by dissolving chitosan in 1% (v/v) acetic acid solution at concentration of 2mg/ ml;

b) preparation of anionic tripolyphosphate (TPP) solution by dissolving TPP in distilled water at the concentration of lmg/ ml;

c) addition of TPP solution into chitosan solution dropwise at 1:2 ratio;

d) formation of chitosan colloid nano particles under mild agitation in room temperature condition;

e) formulation of colloid chitosan nanoparticles at room temperature which was centrifuged at 35,000 rpm for 1 hr.;

f) discarding of supernatant and re-dispersing of the deposit in distilled water; and

g) final collection of the alginate coated chitosan nanoparticles.

8. The method as claimed in claim 3 and claim 7 wherein said loading of OmpA protein to said chitosan nanoparticles comprises the steps of:

a) re-dispersion of colloid said chitosan nanoparticles in 25 ml of distilled water at concentration of 2mg/ mL under continuous ultrasonication to disaggregate the nanoparticles;

b) incubating OmpA protein obtained as a product from the *E. coli* BL2 1 (DE3) with said nanoparticles under mild agitation at room temperature condition for 15 minutes to make final concentration of OmpA protein 1 mg/ mL;

c) loading of OmpA protein subunit to the chitosan nanoparticles by incubating OmpA with said nanoparticles under mild agitation for 15 minutes in room temperature condition;

d) dropwise addition of OmpA loaded chitosan nanoparticles with pH 5.1 to the sodium alginate solution under mild agitation at pH 7 for 10-20 minutes; and

e) final collection of the alginate coated chitosan nanoparticles into calcium chloride ($CaCl_2$) aqueous solution at concentration of 0.524 mmol/L to crosslink the alginate layer, presents on the surface of the nanoparticles.

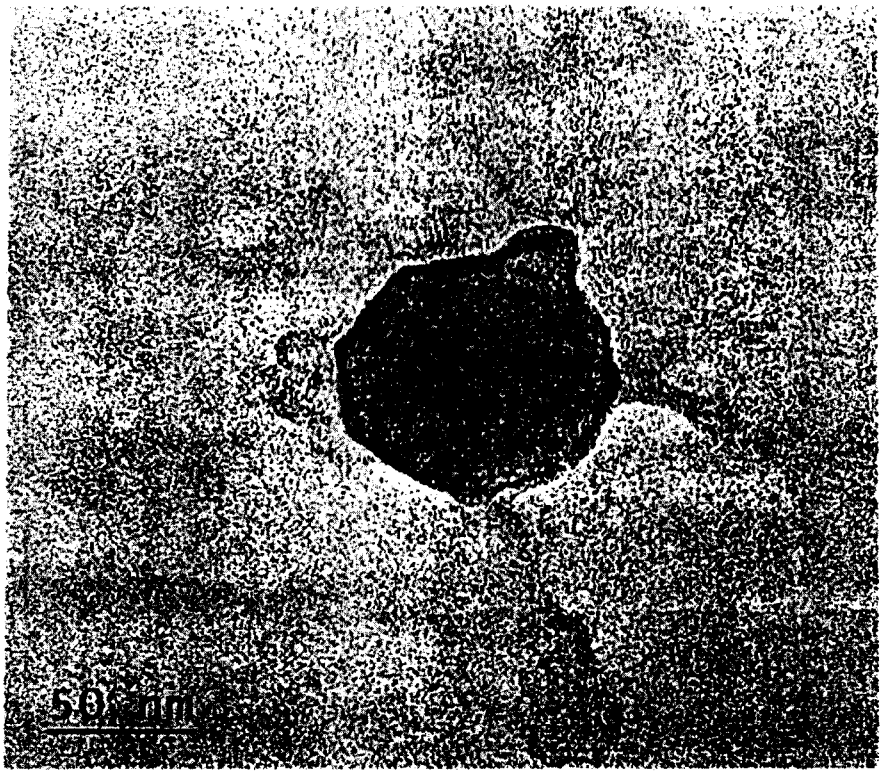

Figure : 1

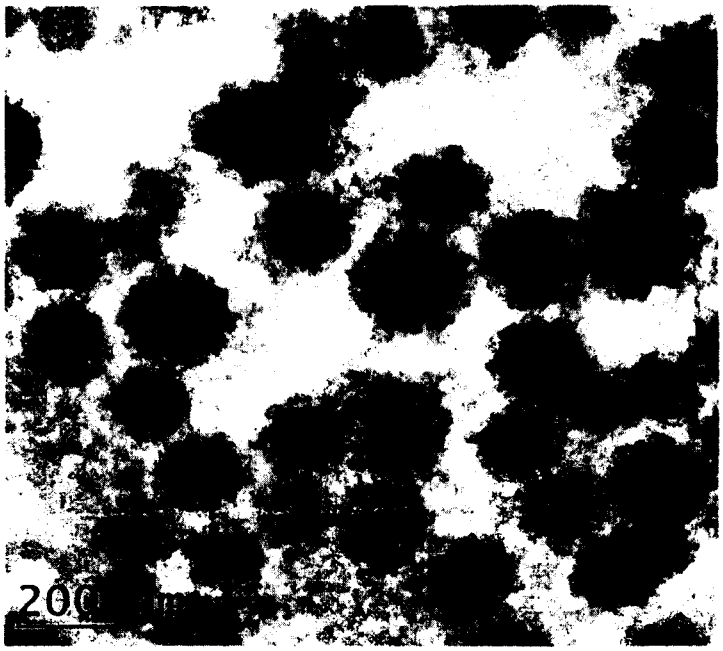

Figure : 2

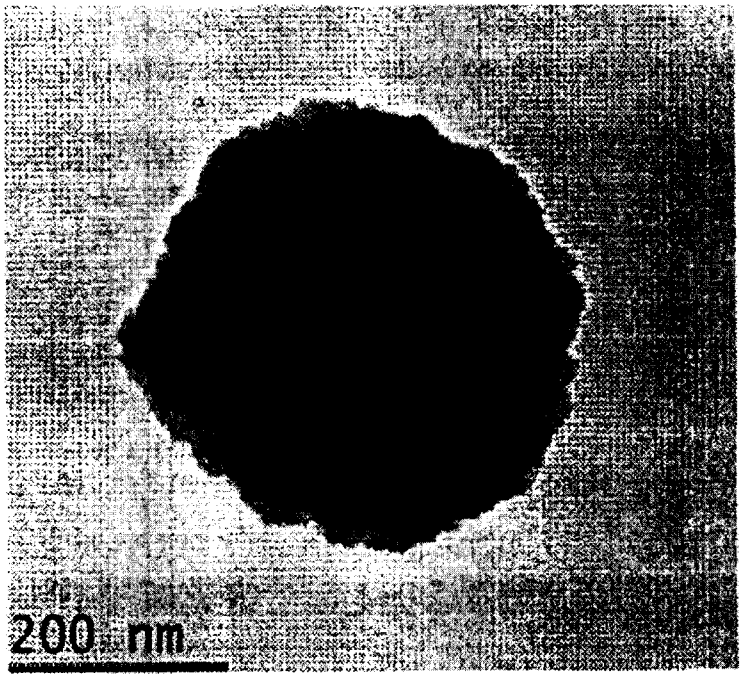

Figure : 2a

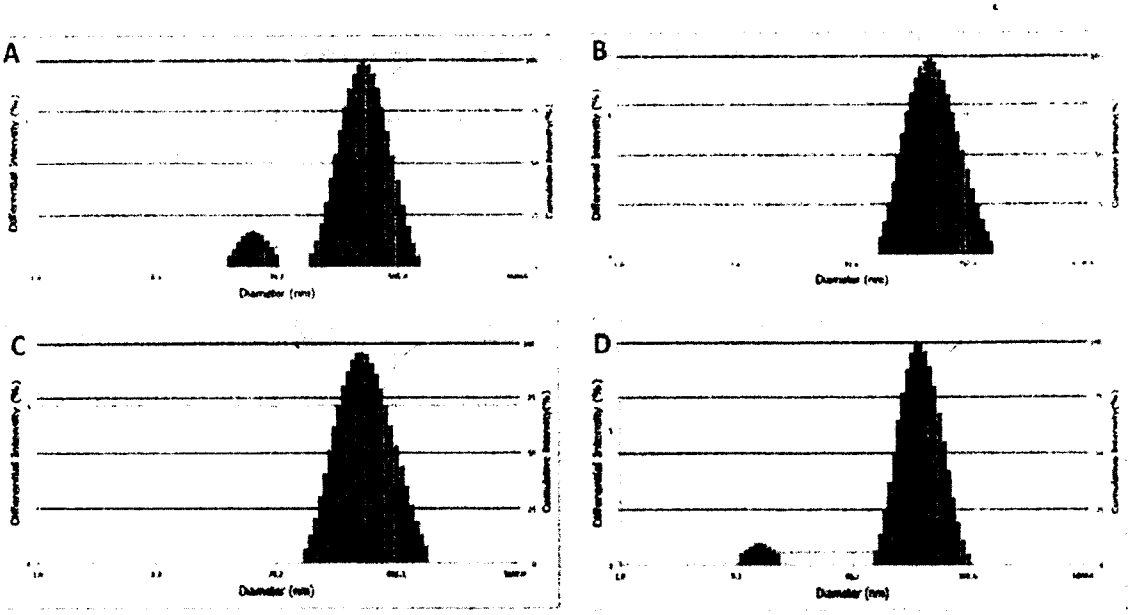

Figure : 3

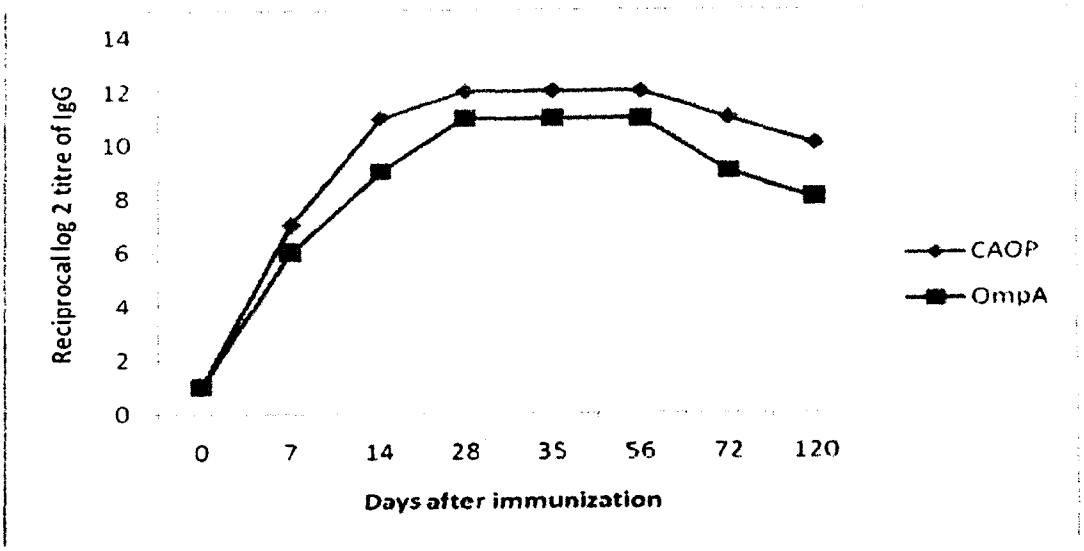

Figure : 4

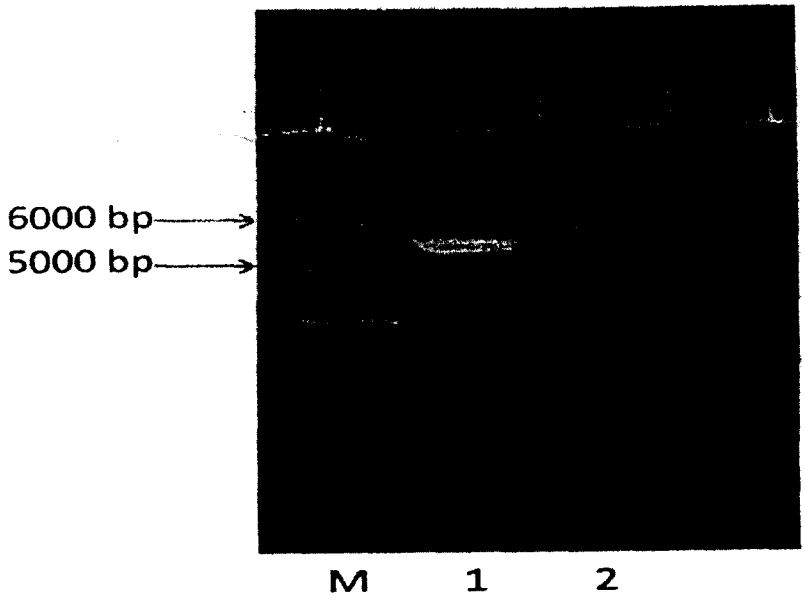

Figure : 5

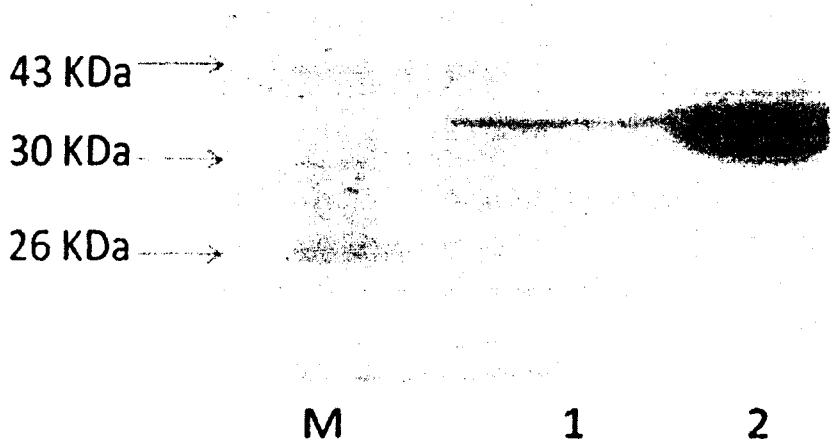

Figure : 6

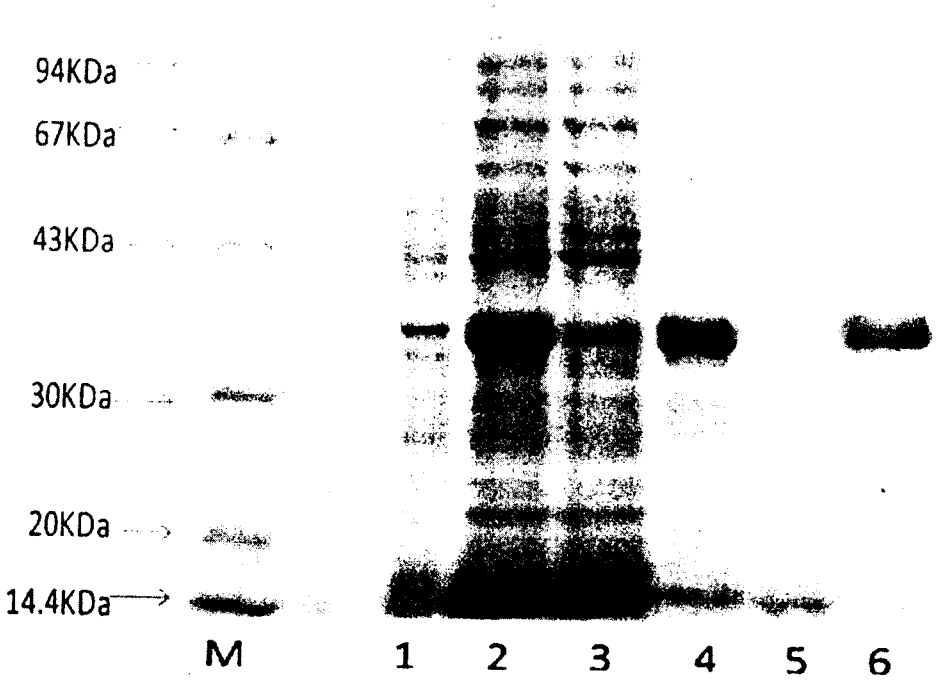

Figure : 7

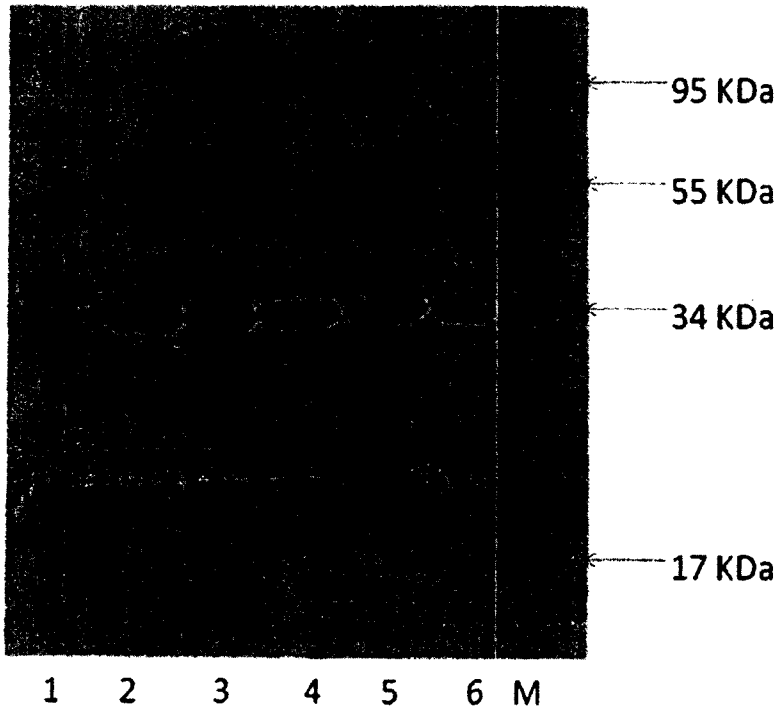

Figure : 8