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Preparation of Pharmaceutical molecules as Vaccine derived from enhanced expression of Hepatitis B surface antigen in Transgenic Potato Callus

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Abstract

Edible vaccine might be solution to get rid of various ailments as it has more advantages compared to traditional vaccine. Vaccines are used most widely in the world also reduced mortality rate caused by various infectious organisms and their use has been considered one of the safe and effective measure and to control various infectious diseases. In the present study, high-level expression of recombinant Hepatitis B surface Antigen (rHBsAg) gene PreS2-S was achieved in yeast *Pichia pastoris*. A single copy of HBV *M* gene (*PreS2-S*) was cloned in PICZA vector thereby rHBsAg protein could then be expressed by induction with methanol. Transformation can be carried out using *Agrobacterium* T-DNA vector, high levels of expression PreS2S-21 (843 bp) can be achieved by using strong specific plant promoters. M-gene was amplified by PCR. The plasmids were transformed into potato callus via *Agro bacterium tumefaciens*. Potato calluses were developed as mass culture on MS medium supplemented with growth hormones. The production of vaccines in callus cells was analyzed by preparation of protein and immunized in to mice and confirmed the level of protein expression from potato callus by ELISA. Purified rHBsAg protein size was confirmed by SDS PAGE and the molecules were visualized by using Atomic Force Microscopy and determined to be organized in 20-25 nm particles. Immunogenicity of purified rHBsAg proteins was demonstrated in mice which were shown to produce specific anti-HBsAb response. The use of plant derived vaccines may overcome some of the major problems encountered with traditional vaccination against infectious disease.

Keywords: Edible vaccine, Hepatitis B surface antigen, *Pichia pastoris*, PCR, Transgenic callus

Contents

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1. Introduction

Vaccines are primary tools in programmers of health intervention for both humans and animals. Vaccines have been revolutionary for the prevention of infectious diseases. During the past decade, advances in molecular immunology have led to development of effective vaccination programmers. However, the cost of production of vaccines by traditional fermentation based systems is high and is hampering progress in this direction. For more than 200 years, vaccination has dominated as the greatest asset for eradication of infectious diseases. With the rising prevalence of antibiotic-resistant bacterial strains and an alarming increase in new and reemerging pathogens, the need for global vaccination continues to remain a high priority. Significant drawbacks in the safety of injectable vaccines hinder their effectiveness.These limitations include the potential for allergic responses to animal proteins co-purified during vaccine harvest, such as influenza virus grown on chicken eggs. In addition, many parenteral (injected) vaccines contain toxic preservatives such as formaldehyde, thiomersal (a mercury-based compound), and aluminum phosphate [1].

More importantly, the need for trained personnel, refrigeration, costly development, shipping expenses, and the risk associated with subcutaneous delivery and needle stick injury makes conventional vaccines impractical. These obstacles especially pertain to impoverished areas of the world, where infectious diseases remain endemic.A new approach to generating improved mucosal vaccines has been advancing during the last decade and involves the application of genetically modified plants. The employment of edible transformed plants as production and delivery vehicles for immunogenic peptides is providing the basis for a dramatic breakthrough in vaccine protective efficacy.

Plants can serve as bioreactors for the production of functional antibodies used in immunotherapy [2]. Edible plants offer a variety of unique approaches for the treatment and prevention of diseases. Foremost, they enable the production of vaccines that would otherwise be ineffective if not delivered to mucosal regions, primarily the digestive tract. Plant tissues containing vaccine proteins can be directly consumed with little or no preparation. During digestion, proteins that elicit an immune response are gradually released onto the vast surface area of the digestive tract, where antigen uptake occurs. Plant species indigenous to specific geographical areas (e.g., banana, potato and rice) can be selected for vaccine production. However, difficulties remain at the level of transformation of certain crops. Ultimately, food plants can be selected for transformation based on hardiness, taste, nutritive value, protein content, and availability, once vaccine effectiveness has been established in model plants that are easier to transform. The adaptation of palatable foods for vaccine delivery will help to increase compliance rates, especially among children. Although plant-based vaccines have enormous potential for protection against disease, little is known about optimum dosage, frequency of inoculation, or the long-term consequences of consuming plant-generated pathogen proteins.

The relative safety of plant-based vaccines compared with vaccination with killed or attenuated whole pathogenic viruses/bacteria reduces the need for medically trained personnel to administer the vaccines. These factors are especially useful in countries lacking sufficient economic resources and health care facilities. In addition, transgenic plant seeds can be easily shipped and stored for long periods of time and do not require refrigeration. Plant-derived vaccines are free from animal pathogen contaminants and may also cut the use of preservatives. Because the viral and bacterial recombinants used to transform plants are not known to invade mammalian cells, further biological safety is assured. A recent exception was brought to attention when *Agrobacterium tumefaciens* was shown to transform mammalian (HeLa) cells [3]. However, the likelihood of genetically modified *Agrobacterium* escaping the laboratory environment is unlikely, due to the destruction of the bacteria by antibiotics following gene delivery to the plant. Bananas are particularly appealing as vaccines because they grow widely in many parts of the developing world, can be eaten raw and are liked by most children.

Hepatitis is an inflammation of liver commonly caused by viral infections. There are five main types of Hepatitis viruses infecting liver, namely, Hepatitis A, B, C, D and E. Hepatitis A and E are transferred enterically; whereas hepatitis B, C and D are transferred through parenteral contact with infected body fluids and may progress into chronic stage of inflammation. Discovery of Australia antigen by [4] (later known as hepatitis B surface antigen) and its relationship with hepatitis B virus (HBV) in early 1970s led to development of diagnosis tests for hepatitis B. Jaundice (yellowing of the skin and eyes), dark urine, extreme fatigue, nausea, vomiting and abdominal pain are among the symptoms of Hepatitis B [5] .Worldwide, more than 2 billion people have been infected with the virus and about 400 million of these live with the chronic infection. Chronic hepatitis B patients may be asymptomatic, but can spread the infection and are at high risk of developing fatal diseases such as cirrhosis and hepatocellular carcinoma [6]. An estimated number of 200,000 and 300,000 chronic Hepatitis B virus carriers die each year from cirrhosis and hepatocellular carcinoma, respectively. Incidence of developing chronic hepatitis B is higher in individuals infected by perinatal route (90%) or during childhood (20- 30%) [7].

Hepatitis B surface antigen (HBsAg) is the first detectable antigen in the serum during the course of infection and for that reason, it is routinely used to screen for the disease [8]. In adult infection, immunotolerant phase marks the incubation period of the acute disease. Depending on the age of the patient and route of Hepatitis B virus acquisition, immune tolerance to the virus is lost and the immune system attacks on the infected hepatocytes. The human hepatitis B virus (HBV) is a partially double-stranded DNA virus of the Hepadnaviridae family. Major and minor subtypes of Hepatitis B virus have been serologically identified at the hepatitis B surface antigen (HBsAg) level.Hepatitis B surface antigens are suitable candidates for vaccine development because of their role in viral clearance [9].

In the present study, an attempt was made towards the production of edible vaccine by expressing Hepatitis B surface antigen in potato callus culture through *Agrobacterium* mediated gene transfer techniques. High-level expression of recombinant Hepatitis B surface Antigen (rHBsAg), PreS2-S was achieved in the methylotrophic yeast, *Pichia pastoris*. Which can be used as expression vector for this purpose, a single copy of HBV *M* gene (*PreS2-S*) was inserted at the downstream of the alcohol oxidase (*AOX1*) promoter of the pPICZA vector. rHBsAg protein could then be expressed in callus of potato via *Agrobacterium* based gene transfer system. High level of rHBsAg expression was obtained it was verified by the atomic forcemicroscopy that rHBsAg has been produced in the desired conformation.

2. Materials and Method

Maintenance of Bacterial and Yeast Strains

Pichia pastoris is an efficient system for the expression of heterologues proteins which combines the advantages of both prokaryotic (high expression levels, inexpensive growth media and easy scale up) and eukaryotic systems (posttranslational modifications) [10]. Moreover, the problems encountered with the other widely used recombinant host *Saccharomyces cerevissia* such as mitotic instability of recombinants and overglycosylation are not practiced [11]. *P. pastoris* strains were grown in Yeast Extract Peptone Dextrose (YPD) medium for seed culture preparation and Yeast Extract Peptone Dextrose Sorbitol (YPDS) medium for cloning purposes. YPDS was supplemented with 100µg/ml Zeocin, whenever required. Buffered Minimal Glycerol (BMGH) and Buffered Minimal Methanol (BMMH) medium were used for expression studies. *P. pastoris* were routinely grown at 30°C unless otherwise stated. Cultures grown on YPD or YPDS agar plates were stored at 4°C, for short term (up to one month) preservation. For long term storage, yeast colonies grown on YPD plates were suspended in 50% glycerol, and stored at -80°C. *E. coli* DH5α strain was routinely grown in Luria Broth (LB) or Low Salt Luria Broth (LSLB) medium at 37°C. Cultures grown on Luria agar (LA) or Low Salt Luria Agar (LSLA) plates were stored at 4°C, for short term (up to one month) preservation. For long term storage, cultures grown until mid-log phase in LB or LSLB were preserved in 50% glycerol, at -80°C. LA and LB were supplemented with 100 µg/mL ampicilin, whenever required. LSLA or LSLB were supplemented with 100 µg/mL zeocin, whenever required.

Plasmid Isolation

Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were mostly used for isolation of *E. coli* plasmid DNA as specified by the manufacturers.0.8% agarose gel was prepared in TAE buffer and run at 90 Volts for 45-60 min after the samples are loaded. The gel was stained in TAE buffer containing ethidium bromide at a final concentration of 0.5µg/mL for 15 minutes. DNA bands were visualized on a shortwave UV transilluminator and photographed by Gel Imaging System. *Pst*I digested lambda DNA marker was used todetermine the molecular weights of DNA bands.

Polymerase Chain Reaction (PCR)

PCR mixture contained 5 µL 10X PCR buffer, 5 µL of 10X $MgCl_2$, 1 µL 10mM dNTP, 10 pmols of each primer, 0.1 µg template DNA, 5 Units of Taq polymerase and sterile dH_2O to complete the volume to 50 µL. PCR conditions are initial denaturation:1 min at 94 ^oC; Denaturation:1 min at 94 ^oC; Annealing:1 min at 55 ^oC;Extension:1 min at 72 ^oC andFinal extension:10 min at 72 ^oC

35cycles.Two primers were used for PCR amplification: MOF; forward primer:(5'gggtaccag**aacaata**atgcagtgg3') and MOR reverse primer: (5'ttccgcggtgaattgaagctt 3'). Restriction sites, *KpnI* and *SacII* are shown underlined.KOZAK sequence is shown in bold.

Vector Construction: Preparation of *E. coli* DH5α Competent cells described by Sambrook *et al*., 1989 was used with slight modifications competent *E. coli* cells were transformed, in *P. pastoris* cells by electroporation the following protocol was used with slight modifications [12].

Expression of the *M* gene in *P. pastoris*

Expression studies in recombinant *P. pastoris* were done according to the instructions of invitrogen. *P. pastoris* glycerol stock was grown on YPD plates for 3 days at 28-30°C. 50 ml of BMGH medium was inoculated with a single colony of *P. pastoris* grown on YPD plates and incubated overnight at 30°C to OD600: 2-6 (with appropriate dilution), which corresponded to the logarithmic phase of growth. Cells were grown at 30°C with constant shaking

at 300 rpm for five days and methanol (100 %) was added to a final concentration of 0.5% every 24 h until the end of the experiment. Cells were centrifuged at 3000 g, 15 min at +4°C and cell pellet was frozen in liquid nitrogen and stored at -80°C.Scale up studies was performed by increasing the culture volume in shake flasks. Protein extraction was performed as described [13] with slight modifications. Cell lysate was centrifuged at 6000 rpm for 10 min at 4°C and supernatant was frozen with liquid nitrogen and stored at -20°C for further analysis.

Preparation of protein:

For the extraction of proteins, Dyna-mill Bead Agitator was used at the Central Laboratory of METU. Cell pellet frozen at -80°C was dissolved to OD600:100 with 300 ml lysis buffer and agitated under the following conditions: 2950 rpm, 10 min, 4°C. Cell lysate was centrifuged at 6000 rpm for 10 min at 4°C the supernatant was frozen in liquid nitrogen and stored at - 20°C till purification. SDS polyacrylamide gel electrophoresis (PAGE) was used for separation of extracted protein and the novel protein size was identified compared with molecular marker. Proteins were stained by Silver Staining as described by [14] and protein concentrations were measuring by using [15].

Plant transformation:

Embryogenic cell culture of potato were established from three month old shoot tip derived callus obtained by culturing shoot tip sections on [16] medium supplemented with 2,4-D (20µM), activated charcoal (0.1%) and Zeatin (2µM) Embryogenic calluses were developed through subsequent subculture methods and maintained the calluses for 4-6 months. The cells were sub- cultured every 10 days and actively growing ones were used for transformation.

Expression of hepatitis B surface antigen gene in Plants via stable Transformation:

Genetic Transformation of plants has facilitated the study of plant gene expression and Hepatitis-B foreign proteins have been successfully expressed in plants. Transformation can be carried out using *Agrobacterium* T-DNA vector, high levels of expression PreS2S-21 (843 bp) can be achieved by using strong specific plant promoters. M-gene was amplified by PCR and cloned into vector containing the strong, constitutive 35SCaMV promoter and a reiterated 35S enhancer. The plasmids were transformed into potato callus via *Agrobacterium tumefaciens*. Potato calluses were developed as mass culture on MS medium supplemented with 0.05 mg 1-1 of indole acetic acid, 7 g 1-1 of agar, 100 mg 1-1 of kanamycin, 250 mg 1-1 of cefotaxin. Regenerated callus were segregated and sub culture in fresh medium and transgenic plantlets were multiplied in vitro.

Analysis of protein from Potato callus:

Protein was extracted from callus tissue as described earlier (Mason et al 1992). The total soluble protein from transformed and non transformed cells was separated on a 12% SDS –PAGE [17] and silver stained. Total soluble proteins were extracted from 1 g of callus tissue and the resulting extract was concentrated to 100 by freeze drying in low mode in a speed vac. The soluble protein was re-extracted into 20mM phosphate buffer pH 7.4 using centricon columns (Amicon) and 20 µl of the sample was used for western analysis. Sample was used for western analysis with anti-HBsAb antibodies was used as a primary antibody and rabbit anti-mouse IgG peroxidase conjugate (sigma) as a secondary anibody (2:20,000dilution). The reaction was developed by DAB (diaminobenzidine-H_2O_2) solution (Sigma). Western blot was carried out as described [18].The amount of Potato total soluble protein content was estimated by a Bradford assay (Hurst 1991). Comparison of total soluble protein of the induced and non induced Potato cells were used to estimate the recombinant HBsAg expression levels**.**

Mice Immunization:

Inbred Swiss Albino mice (20-25g) were maintained in the Laboratory Animal Feeds, (Bangalore) and albino mice were maintained in the laboratory with free access to commercially available food pellets and water. For each experimental group 4-5 mice aged 10-12 weeks (25-30g) were used. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions [Temperature 27^0C and 12 hours light/dark cycle] throughout the experimental period. Animal experiments were carried out following the guidelines of the animal ethics committee of the Institute. The recombinant HBsAg protein was adsorbed onto Al $(OH)_3$ gel for one hour, at room temperature with constant stirring. To evaluate immunogenicity mice were immunized subcutaneously with 0.5 ml of this preparation. Vaccination was repeated in 15 days intervals for twice. Serum was collected after one month from the initial vaccine administration and pooled in a single tube. Control animals were immunized with control callus extract (untransformed). Serum samples were collected at several time intervals from tail blood. Antibody determination in serum was done by ELISA.

Enzyme Linked Immunosorbent Assay (ELISA)

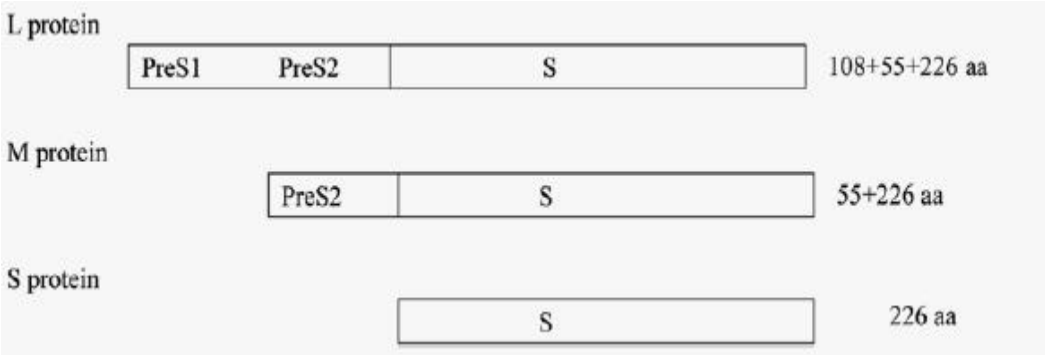
Dia.pro HBsAb commercial ELISA plates coated with HBsAg from human origin were used for ELISA. Mouse sera (control negative serum, control positive serum and test serum) was added to plates and incubated for 2 h at room temperature. The plate was washed with saline containing 0.05% Tween 20 (washing solution), for three times. Alkaline phsopatase conjugated, goat anti-mouse IgG (Sigma) was diluted with the PBS, containing 2% fetal calf serum and 0.05% Tween 20 and added to plates at 1/15000 concentration. The plate was incubated for two h at room temperature and washed with the washing solution for three times. Biorad AP Substrate was prepared according to manufacturer's instructions and added to the plates. Colour development was observed to judge for the positive or

negative results. 100 µl working volume was used during the assay. Antibody titers were calculated as the dilution of the sample giving an extinction were measured at 450nm

3. Results and Discussion

Hepatitis B envelope proteins are synthesized at endoplasmid reticulum and fold by forming disulfide linked homo and heterodimers. HBsAg is found to contain 14 cysteine residues. Reduction and alkylation of the disulfide bonds results in complete loss of antigenicity, however reduction alone doesn't effects serological activity of the protein. Therefore, it is assumed that conformation of antigenic determinant is determined by disulfide bonds [19]. Hepatitis B envelope is composed of three related surface proteins; namely small (S), middle (M) and large (L) surface antigens. They are encoded by a single open reading frame (ORF) called E containing 389 to 400 amino acids depending on the genotype [20]. The large surface protein is the translation product of the entire ORF (389 to 400 a.a.), the middle surface protein lacks 119 a.a. from the N terminal of L protein (the preS1 sequence) and the small surface protein lacks 55 a.a. of M protein (the preS2 sequence). The pre-S1 region directly interacts with hepatocytes, the pre-S2 region has been implicated in the polymerized albumin mediated interaction and the S region contains the complete information for self assembly in the absence of capsids to form higly antigenic subviral particles.

Structure of the hepatitis B surface proteins



Six protein products are derived from the open reading frame: a glycosylated and nonglycosylated form of the major protein translated from the S domain only; middle protein having one or two polysaccharide chains translated from preS2 and S region and the glycoslated and non-glycosylated form of the large protein encoded by the entire length ORF All three proteins are glycosylated at N146 residue, except for gp36 (M protein) which is glycosylated atN4 residue. Hepatitis B surface antigens are suitable candidates for vaccine development because of their role in viral clearance

Cloning of *PreS2+S* gene in *P. pastoris*

HBsAg *PreS2+S* gene (*M* gene) was amplified from pS2-S/pIL2 plasmid by PCR. MOF and MOR primers, were used in the reaction and the expected band size of 864 bp (843 bp from *M* gene and 21 bp from primer added restriction digest and KOZAK sequences) was obtained as shown in [Figure 1].

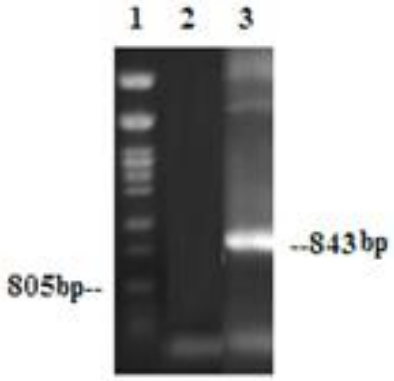


Figure1: PCR amplification of *PreS2-S*

Legend: Agarose gel shows PCR product: Lane 1: λ Pst Marker, Lane 2: Negative control; Lane 3: *M* gene (843 bp) amplified by PCR.

Amplified fragments were purified from agarose gel using Qiagen Gel extraction kit and ligated with pGEMT**R** sub-cloning vector. Ligation product was transformed into competent *Escherichia coli* DH5α cells and recombinants were selected on X-gal + IPTG + ampicillin containing LA plates. Plasmids were isolated from putative recombinant white colonies and presence of insert was verified by double digestion of restriction enzymes, *Kpn*I and *Sac*II (Figure 2).

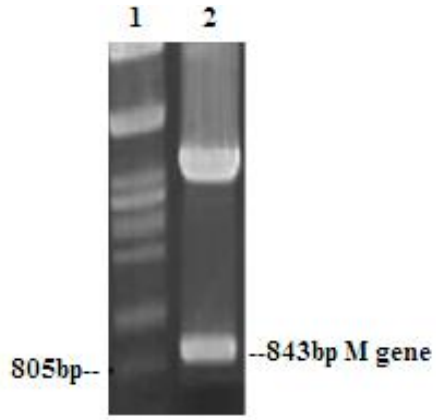


Figure 2: Confirmation of insert size on agarose gel

Legend: Restriction analysis of pGEMTR clones. Lane 1: λ Pst Marker. Lane 2: *Kpn*I and *Sac*II digested pGEMTR vector carrying *M* gene.

For cloning into *P. pastoris* expression vector (pPICZA), both this vector and the pGEMT vector carrying *M* gene were digested with *Kpn*I and *Sac*II enzymes and ligated after purification from agarose gel. Ligation product was cloned into *E. coli* DH5α cells and recombinants were selected on LSLB plates with zeocin. Plasmids were purified from the recombinants grown on selective plates and the presence of the gene was verified by PCR. For transformation into *P. pastoris*, the pPICZA plasmid containing the *M* gene was linearized with *Sac*I enzyme, the site of which is in the 5' *AOX1* region. Linearized plasmid was then transferred into *P. pastoris* strain GS115, gene insertion event was expected to occur at *AOX1* loci and either one of the two *AOX1* regions on the pPICZA vector by homologous recombination. There were two bands on agarose gel for recombinant colonies. Parent plasmid pPICZA gave rise to the amplification of 1158 bp PCR product (846 bp gene + 325 bp from the plasmid) and 2.2 kb corresponding to the*AOX1* gene Figure 3). The transformation efficiency has increased up to 25 fold when yeast cells were treated with 0.1M LiCl and 10mM DTT before electroporation.

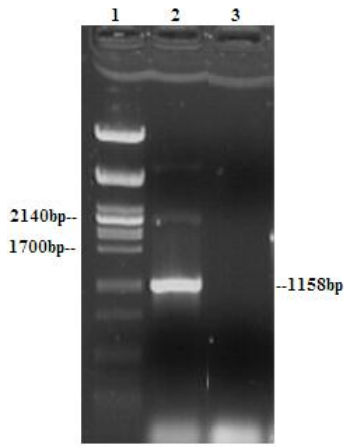


Figure 3: Confirmation of transformant of *P. pastoris* by PCR.

Legend: Lane 1: λ Pst Marker. Lane *2*: PCR product of pS2-S/pIL2 plasmid using MOF and MOR primers. Lane 3: PCR negative control.

The presence of two bands on agarose gel showed that the *AOX1* gene was not disrupted so that the resultant colonies were all Mut+. HBsAg particles can be fold in the desired 22 nm conformation more efficiently in Muts strain when compared to Mut+ strain. This is assumed to be due to slow growth of Muts strain which prevents aggregation of HBsAg particles to a larger extent.

Expression of PreS2+S gene in *P. pastoris*

HBsAg protein has been produced in nonglycosylated form in *P. pastoris* expression system. M protein was 32 kDa in unglycosylated form and expected to be expressed as a ~33kDa protein along with 0.84 kDa from 6xHis Tag. Expression of rHBsAg protein in nonglycosylated form (33kDa) has been confirmed by SDS PAGE (Figure 4). Purification of rHBsAg protein by adsorption chromatography and ultra filtration, followed by size exclusion chromatography was also verified through SDS PAGE.

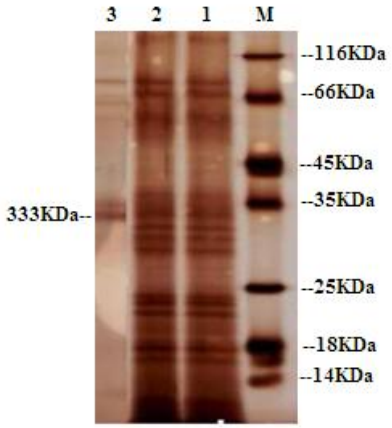


Figure 4: SDS PAGE showed Purifified of rHBsAg protein

Legend: Total protein and eluted protein fraction confirmed its size on SDD PAGE on comparison with standard marker protein. Lane M: Marker protein with various sizes. Lane 1-2: The total protein loaded to size exclusion chromatography. Lane 3: Purified fraction Protein of adsorption chromatography. ELISA results have shown that mice immunized with rHBsAg elicited specific anti-HBsAb. This proved that, recombinant protein has been produced in the desired immunogenic conformation. Mice immunogenicity test was done to check for the immunogenicity of HBsAg purified from the recombinant *P. pastoris*. 60µg/ml protein obtained from the size exclusion chromatography was adsorbed onto Al $(OH)_3$ gel and this preparation was used to immunize mice to evaluate IgG response elicited by the antigen [21]. Production of anti-HBsAb in test animals was evaluated with Dia.pro HbsAb commercial ELISA Kit. The kit was coated with the heat inactivated HBsAg (ad/ay subtypes) obtained from a human origin (plasma). Unvaccinated mice serum was used as a negative control. Mice immunized with Pasteur's Hepatitis B vaccine was used as positive control. The test serum and the control serums were used without dilution. The results of ELISA assay is shown in Figure 5.

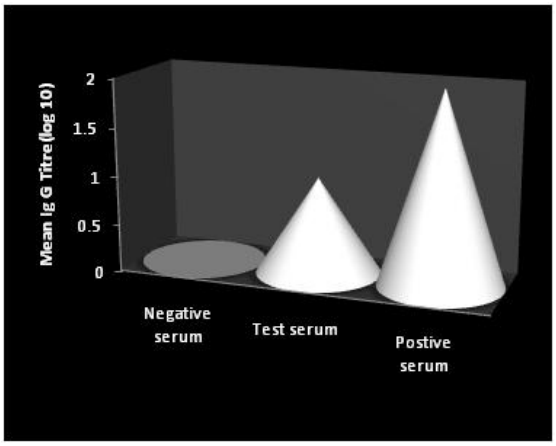


Figure 5: Measurement of IgG level in mice by ELISA

Legend: Specific IgG levels in mice, immunized with rHBsAg (ELISA test). 1: Negative serum (unvaccinated mice). 2: Test serum (anti-rHBsAb). 3: Positive serum (mice immunized with Hepatitis B vaccine).

Atomic Force Microscopy (AFM) images of purified rHBsAg, obtained from size exclusion chromatography were taken by using 'NanoMagnetics Instruments AFM'. Cross Sectional and three dimensional images of HBsAg spherical particles are shown in Figure 6**.** Majority of the particles were between 20-25 nm in size as expected. HBsAg particles were immunogenic both in monomer and particle forms (~100 monomer units). Still, the 22 nm particle form is used in vaccine formulations, as it is known to be 1000 times more immunogenic than HBsAg monomers. Correct folding of the antigen is very important for its recognition by the immune system [22] as verified in the experiment.

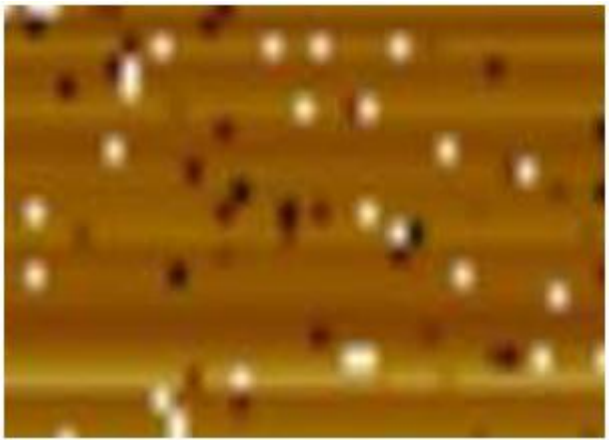


Figure 6: Atomic Force Microscopy image of the rHBsAg particles

Legend: Phase contrast image. Particles marked with white circles, are standing for rHBsAg particles.

HbsAg contains 14 cysteine residues per monomer. It has been compared with previous finding and analyzed during *in vitro* purification of yeast derived recombinant antigen, numerous disulfide bonds form between monomers creating mature particles. Besides these, high number of disulfide bonds formed in the antigen is also responsible for the formation of some heterogeneous (larger) particles, due to intermolecular interactions. In this work, majority of the particles obtained were between 20-25 nm in diameter .However, the average diameter of the particles was around 35 nm because of the homogeneous particle distribution caused by the existence of some larger particles. Formation of larger heterogeneous particles, besides 22 nm particle formation was something expected and observed as previously mentioned. Results showed that rHBsAg protein highly expressed in yeast *P. pastoris* which could be elicited specific anti-HBsAb high level production transgenic callus of potato and the purified product was suitable for immunization purpose.

Plant-derived biopharmaceuticals Products:

Generally, levels of pharmaceutical proteins produced in transgenic plants have been less than the 1% of total soluble protein that is needed for commercial feasibility if the protein must be purified. Plant derived recombinant hepatitis-B surface antigen induced only a low level serum antibody response in a small human study, probably reflecting the low level of expression (1–5 ng g−1 fresh weight) in transgenic potato and expression levels should be increased further for practical purposes. "Recombinant protein product" refers to any protein product expressed from recombinant genetic material encoding amino acids, including peptides, polypeptides, proteins, oligoproteins and/or fusion proteins product would be preferable for therapeutic purpose.

4. Conclusion

Hepatitis B surface antigen *M* gene (*PreS2-S*) was cloned into pPICZA expression vector which was then transformed to *P. pastoris*. Recombinants were verified by colony PCR. Protein product was successfully expressed at a large scale in a batch fermentor, with methanol induction. The purification of rHBsAg was carried out by using various chromatography techniques with specific column on Superdex75. Purified rHBsAg protein size was confirmed by SDS PAGE and molecules were visualized by using Atomic Force Microscopy and determined to be organized in 20-25 nm particles. Immunogenicity of purified rHBsAg proteins was demonstrated in mice which were shown to produce specific anti-HBsAb response. Practical considerations will dictate the choice of biopharmaceutical proteins and the crop in which they are to be produced. These include yield, storage conditions, containment properties, initial set up and running costs, purification strategies, design the of the market, environmental concerns, public perception and competing technologies. Access to several alternative approaches to optimize protein synthesis in plants in an environmentally sound manner augurs well for the safe production of biopharmaceuticals in transgenic plants and for greater availability of these proteins to populations requiring them.

Edible vaccine might be solution to get rid of various ailments as it has more advantages compared to traditional vaccine. It would overcome the problems associated with traditional vaccine like cost, production, distribution and delivery and could be incorporated into the immunization plans. It would be more beneficial and profitable to populations of developing world.

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