Recombinant Hepatitis-B Antigen (rHBsAg) Characterization

Hepatitis refers to the inflammation and damage of liver tissue mostly caused by viral infection. Excessive use of drugs or alcohol, infections, autoimmune diseases, and non-alcoholic steatohepatitis can also cause hepatitis.

Depending upon the type of viruses, there are five main types of viral hepatitis: type A, B, C, D, and E. Hepatitis A and E are typically caused by ingestion of contaminated food or water. Hepatitis B, C and D usually occur as a result of parenteral contact with infected body fluids. All type of viruses can cause acute infection. Type B and C can particularly lead to chronic disease. Hepatitis A, B, and D are preventable with immunization.

Hepatitis B viral infection is a major global health problem which can lead to chronic infection and puts people at high risk of death. Globally, an estimated 257 million people are living with Hepatitis B virus infection, defined as Hepatitis B surface antigen positive (HBsAg). *Ref: www.who.int/news-room/fact-sheets/detail/hepatitis-b*

Brief History of Hepatitis B Vaccine (HB) Development

The first ever blood-derived hepatitis vaccine was developed by Virologist Maurice Hilleman at Merck, who hypothesized that human immune system can synthesize specific antibodies when injected with hepatitis B surface protein. He devised a multistep process to purify the infected blood so that only the hepatitis B surface proteins remained. Thus 'Heptavex' the first-generation blood derived vaccine was developed and approved in 1981. It was later withdrawn from the marketplace in 1986 when Pablo DT Valenzuela, Research Director of Chiron Corporation, succeeded in making the antigen in yeast and invented the world's first recombinant vaccine.

Recombivax HB manufactured by Merck Sharp & Dohme, was approved for marketing in West Germany and was later approved by the United States Food and Drug Administration in 1986. This was quickly followed by two similar recombinant hepatitis B vaccines: Engerix-B, which was marketed by SmithKline Biologicals, approved in Belgium in December 1986 and in the US in September 1989; while GenHevac-B was manufactured by Pasteur Vaccines and was approved in France in May 1989. These vaccines are still in use today. In 2017, almost after 28 years, FDA approved a new two-dose hepatitis B vaccine for adults - HEPLISAV-B.

Hepatitis B Virus and Antigen Structure

Hepatitis B virus (HBV) is an enveloped, partially double-stranded DNA virus of the *Hepadnaviridae* family. The infectious Hepatitis B virion, known as the Dane particle, is approximately 42 nm in size and is composed of an outer lipid envelope containing viral glycoproteins as well as an inner nucleocapsid (Figure 1).


Saba Naaz Siddique
Director-Sales
saba@shantani.com


Anuja Mahamunkar
Sr. Research Associate


Shraddha Pote
Sr. executive-BD
spote@shantani.com

The viral glycoproteins within the lipid envelope of the virion constitute the Hepatitis B surface antigen (HBsAg). The HBsAg include a major polypeptide of 226 amino acids (aa) designated as small HBs (SHBs) previously also called major surface protein, in a non-glycosylated (p24) and glycosylated (gp27) form. The middle-sized protein (MHBs), which shares the 226 aa of the p24 region at the C terminus and has an additional 55 aa residue at the N terminus, is termed pre-S2 corresponding to gp33 and gp36. The large HBs protein (LHBs) contains, in addition to the S and pre-S2 domains, the pre-S1 domain of 119 aa (p39, gp42)[1,2,4–6]. In the native envelope, all the proteins SHBs, MHBs, and LHBs are covalently linked to one another by intermolecular disulfide bonds between the S domains and partially embedded in membrane lipids. Ref: *Shouval, Daniel., Hepatitis B vaccines. Journal of Hepatology, Volume 39, 70 – 76 (2003)*

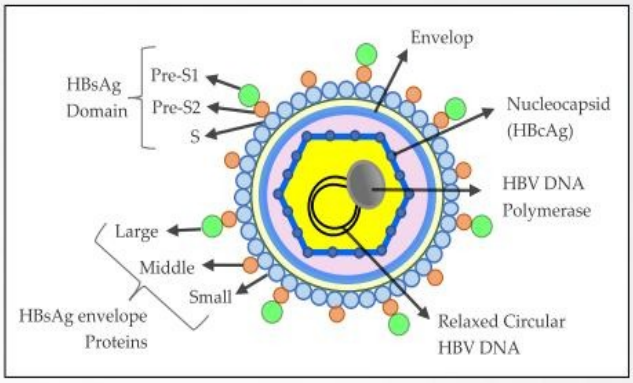

Fig. 1: Hepatitis B Virion.

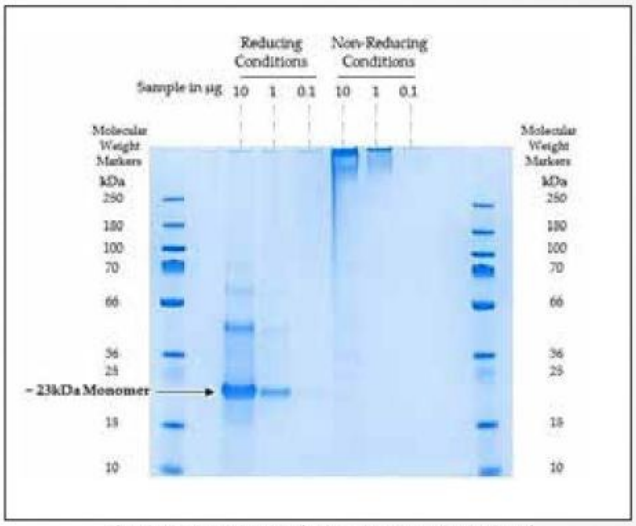

Fig. 2: SDS-PAGE Analysis - Coomassie Stained Protein Bands of rHBsAg.

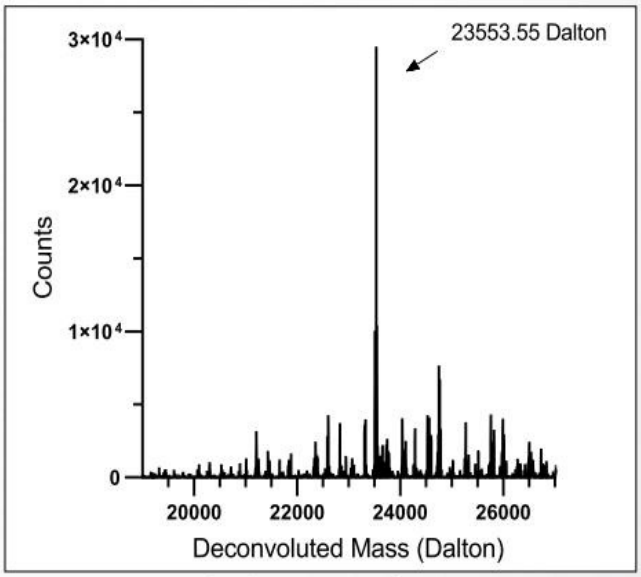

Fig. 3: Deconvoluted Intact Mass of rHBsAg Monomer.

Need for Characterization

The development of vaccines has an immense impact on the overall world health condition. Consistent global efforts are being made to develop safe, effective and affordable vaccines. Over the past few years, the level of scrutiny for biopharma products has been increased tremendously. Establishing safety and efficacy of the drug product through extensive physico-chemical characterization, has therefore, become increasingly important in bringing new vaccines to the world market.

Technology solution providers like 'Shantani', that provides one-stop solution for fulfilling the program's regulatory requirements, supports biopharma product developers worldwide through their expertise in physico-chemical characterization of biopharma products.

In this segment, we are showcasing a characterization case study which was performed at Shantani to establish the Purity, Identity and Integrity of recombinant Hepatitis B Antigen (rHBsAg) using standardized analytical workflow.

1. Single Dimension Gel Electrophoresis (1-D SDS-PAGE)

Methodology

Three different concentrations of reduced and non-reduced samples (0.1 µg, 1.00 µg and 10 µg) were prepared. For reduction, protein samples were incubated with 10 mM DTT at 90° Centigrade for 10 minutes. In non-reducing conditions, samples were heated at 95° Centigrade for 10 minutes and centrifuged. Clarified samples were separated using 4-12% gradient gel. Separated proteins in SDS-PAGE

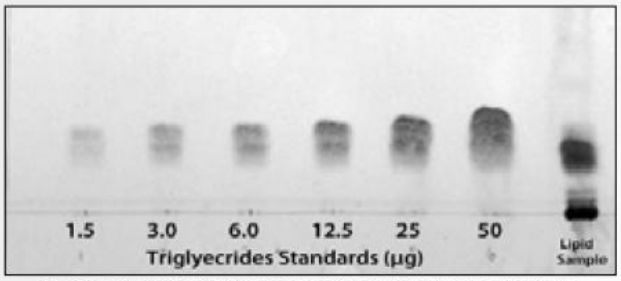

Fig. 11: Analysis of Triglycerides in Lipid extracts of HBsAg.

S. No	Lipid Class	Mean ± SD (% of Total lipid)
1	Phosphatidyl Choline (PC)	62.95 ± 6.46
2	Phosphatidyl Inositol (PI)	3.35 ± 0.87
3	Phosphatidyl Ethanolamine (PE)	1.5 ± 0.42
4	Cholesterol (CH)	0.56 ± 0.01
5	Cholesterol Ester (CHE)	0.09 ± 0.01
6	Triglycerides (TG)	29.47
7	Phosphatidyl Serine (PS)	Not Detected
8	Sphingomyelin (SM)	Not Detected
9	Lyso- Phosphatidyl Choline (Lyso-PC)	Not Detected

Table 2: *Lipid Profile of rHBsAg.*

S. No.	Sample	Cholesterol (% of total extracted lipid)	Cholesterol Ester (% of total extracted lipid)
1.	Replicate-1	0.55	0.09
2.	Replicate-2	0.57	0.08
3.	Replicate-3	0.56	0.10
	Average + SD	0.56 + 0.01	0.09 + 0.01

Table 3: *Measured Cholesterol and Cholesterol Esterase in Different Replicates.*

trypsin and chymotrypsin were sufficient to establish the identity of the protein. An overall coverage map of the identified peptide is shown in Figure 5.

4. Particle-Size Analysis using Differential Light Scattering

Methodology

Particle size of the sample was measured using, Particle Size Analyzer, which operates on the principle that dynamic light scattering can be correlated with the size of the object. 1ml of sample was placed in a clean disposable cuvette. Combined data from three independent acquisitions were analysed to obtain the physical dimensions of particle present in the sample.

Results

The measured baseline index of 9.4 confirmed that the sample was clean and auto-correlation function was not distorted due to large particles or dust particle. For data analysis and representation, DLS volume distribution was preferred over intensity distribution, to avoid misleading representation of the population due to, if any, large size particles (Kee 2009, Strods *et. al.* 2015). The calculated mean diameter of particle was found to be 22.9 nm. Polydispersity of the sample was 0.17 which suggest narrow to moderate distribution of the particle of different size in the sample. Further, size distribution analysis suggested that the median diameter of the particles was 21.4 nm and effective diameter was 30.1 nm and particle distribution is centred around particle size value 21.4 nm (Figure 7). Calculated mean particle diameter of 22.9 nm was in agreement with earlier studies (Zhao *et al.* 2006, Vnek *et al.* 1977).

5. Host Cell Protein Analysis using Western Blot Method

Methodology

Proteins at three different concentrations were reduced and separated on an SDS-PAGE gel. After separation, the gels were completely destained using methanol: acetic acid: water solution (20:10:70). Proteins from the gel were transferred to a 0.45 µm nitro-cellulose membrane using a semi-dry transfer apparatus. To reduce the non-specific interactions, membrane was incubated with 2% non-fat dry milk for 3 hours at room temperature under orbital shaking. Membrane was then probed with either anti-HBsAg or anti-HCP (Host-Cell Protein) polyclonal antibody overnight with constant shaking at 4°C. After primary antibody incubation, membrane was washed 3X with TBST for 5 minutes. To detect the anti-HBsAg or anti-HCP antibody membranes were incubated with anti-mouse secondary antibody or anti-rabbit secondary antibody for 1 hour at room temperature. Membrane was then developed using enhanced chemiluminescence substrate after washing 3X with TBST. Images were captured by Bio-Rad gel documentation system.

Results

Western blot images along with SDS-PAGE separated, CBB stained protein in reduced condition is shown in Figure 7. At 0.1, 1 and 10 µg protein load, anti-HBsAg antibody interacted with monomeric and multimeric forms of HBsAg. When membranes were probed with anti-HCP antibody, a few protein

bands at 10 µg protein load, located at >100 kDa were observed. Observation of protein bands when samples were probed with anti-HCP antibody may suggest presence of HCP in sample. However, considering that no protein bands > 100 kDa were observed in Coomassie blue stained gel, it suggests that the amount of HCP present in the sample is significantly low. It is known that Coomassie stain typically stains protein present in higher nanogram quantity and western-blot methods being very sensitive, can detect presence of protein at picogram quantity. Taken both the factors into account, it was concluded that in terms of protein purity, presence of host-cell protein is negligible in the sample.

6. Extraction and Quantification of Lipids by Gravimetric Analysis

Methodology

Lipids from the sample were extracted using Bligh and Dyer Method (Langley *et al*. 1988).

Results

The amount of lipid and lipid: protein ratio obtained in multiple replicates is compiled in Table 1.

Lipid to protein ratio calculated in this study was 1.2. In earlier studies (Gavilanes et al. 1981; Meeren *et al*. 1994), the lipid to protein ratio was found to be ~ 0.5. To understand the difference, additional experiments were carried out. Using same method parameters and instrument setting, when a large amount (15 mg) of protein was used as starting material the obtained lipid to protein ratio was found to be 0.5. However, when 4.5 mg protein was used as starting material, lipid to protein ratio increased to 1.2. It was concluded that relatively lesser amount of starting material allowed better extraction of lipid from the sample that leads to increased lipid to protein ratio.

7. Quantitative Analysis of Lipids

Methodology

Thin Layer Chromatography (TLC) was used to characterize and quantify the lipid components of HBsAg. Specific solvent system was chosen for different classes of lipid for their better separation and resolution. 5 µg of extracted HBsAg sample were applied as a streak on a TLC plate and allowed to dry at room temperature. For phospholipid separation, the TLC plate was developed in a solvent system containing chloroform / methanol / glacial acetic acid / water mixture in appropriate ratio. For separation of Sphingomyelin and Lyso-PC, a solvent system containing chloroform / methanol / aqueous ammonia / water was used. The TLC plate for Neutral Lipids were developed in a hexane / diethyl ether / glacial acetic acid solvent system. After the solvent front reached the distance of 1 cm from the end of the plate, the plates were removed and stained by spraying with the respective stain solutions. The lipids were stained with molybdenum blue reagent which is specific for phospholipids while the Neutral Lipids stained with phosphomolybdic acid.

S. No.	Glycans	µg/mg Protein
1	N-Acetyl Glucosamine	0.67 ± 0.09
2	Mannose	2.42 ± 0.47
3	Fucose	Not Detected
4	Galactose	Not Detected
5	Glucose	Not Detected
6	N-Acetyl Galactosamine	Not Detected
7	N-Acetyl Neuraminic acid	Not Detected
8	N-Glycolyl Neuraminic acid	Not Detected

Table 4: *Glycan obtained and their concentration in the protein.*

The TLC images were captured using Gel documentation system (Figures 9,10,11,12). A standard concentration curve was constructed using the band intensity values of known concentrations of Phospholipids and Neutral Lipids standards. The linear regression analysis was used to calculate the unknown concentration of the Phospholipids and Neutral Lipids in the samples. Cholesterol and Cholesterol ester was measured by enzymatic method using fluorometric detection.

Results

The summary of the lipid composition of the sample is presented in Table 2.

Phospholipid

The Phosphatidyl Choline (PC) content in the sample was found to be the highest of all the identified lipids. The PC was calculated to be 63% of the total extracted lipids while the Phosphatidyl Ethanolamine (PE) and Phosphatidyl Inositol (PI) were found to be 3.4% and 1.5%. Other phospholipid species such as Phosphatidyl Serine (PS), Phosphatidic Acid (PA), Sphingomyelin (SM) and Lyso-Phosphatidyl Choline (Lyso-PC) were also analysed. However, none of these lipids could be detected with above mentioned method.

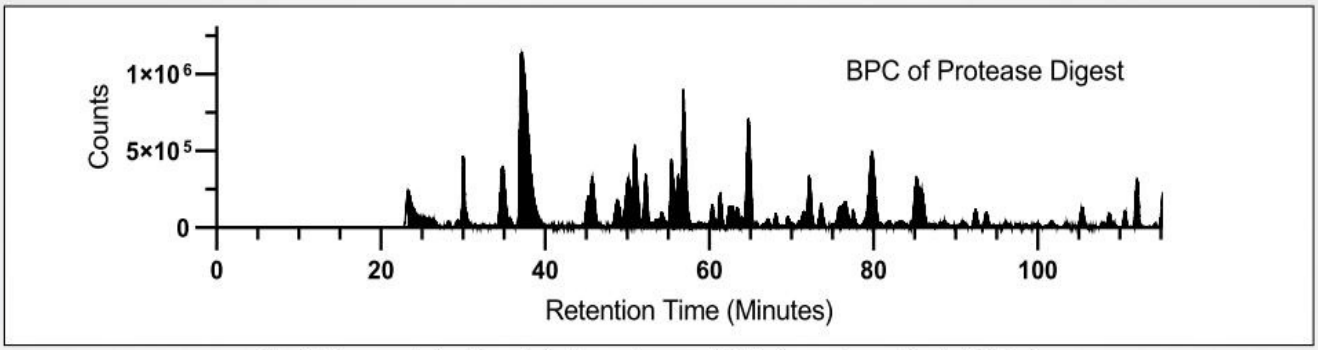

Fig. 4: Representative Base Pair Chromatogram (BPC) of a protease digest of rHBsAg.

MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGSPVCLGQNSQSPTSNHSPTSCPPICPGYRWMCLRRFI
IFLFILLLCLIFLLVLLDYQGMLPVCPLIPGSTTTSTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSWAFAKYL
WEWASVRFSWLSLLVPFVQWFVGLSPTVWLSAIWMMWYWGPSLYSIVSPFIPLLPIFFCLWVYI

Fig. 5: A 100% coverage of known Amino Acid of rHBsAg from Trypsin and Chymotrypsin

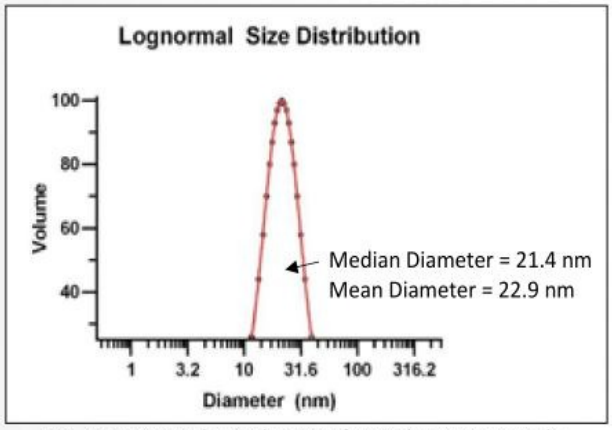

Fig. 6: Lognormal Distribution of Particle Size in Sample.

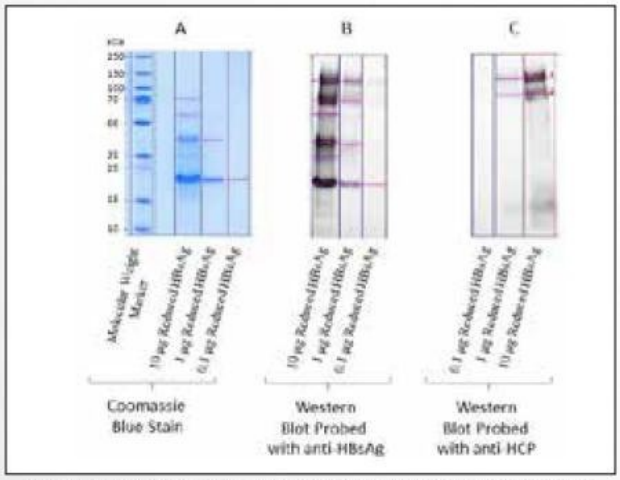

Fig. 7: Host-Cell Protein Analysis using Western-Blot Method.

gel were visualized using CBB dye. Gel images were captured using Bio-Rad gel documentation system and molecular weight of the visible protein bands were calculated using a validated method that utilize molecular weight markers as standards.

Results

In reducing conditions, a predominant protein band between 25 kDa and 15 kDa, and its multimeric bands were observed. In non-reducing conditions, as expected, protein did not enter the gel suggesting the molecular weight of protein is >> 250 kDa. The molecular weight of the monomer, calculated by the Rf values of the protein after applying the correctional factor, was calculated to be in the range of ~23.3 kDa. The molecule weight was found to be similar with the theoretical (Norder *et al*. 1992) and previously reported (Zhao *et al*. 2006,) molecular weight of the monomer. A representative image of CBB stained gel is shown in Figure 2. Based on the understanding that in reducing conditions different bands were either monomer or its multimers, densitometric analysis of gel concluded that sample purity is ≥ 99%.

2. Intact Mass Analysis of Monomer

Methodology

Protein equivalent to 10 µg was separated over SDS-PAGE gel. The major protein band from the gel was carefully sliced, washed and crushed. Proteins from the crushed gel were extracted by overnight incubation of the crushed gel band with protein extraction buffer at room temperature under constant stirring. The ensuing protein solution was concentrated and dissolved in 0.1% Formic Acid. This protein solution was directly infused into an Electrospray based Q-TOF mass-spectrometer. The acquired Mass/charges in the spectra were deconvoluted using MassHunter BioConfirm

S. No.	Sample	Amount of Lipid Extracted (mg)	Protein Amount Used for Extraction (mg)	Lipid / Protein	Lipid / Protein (Average Value and Standard Deviation)
1.	Replicate-1	5.2	4.5	1.20	1.2 ± 0.1
2.	Replicate-2	4.8	4.5	1.07	
3.	Replicate-3	5.8	4.5	1.30	

Table 1: *Lipid / Protein Ratio in Different Replicates*

software, which established the molecular mass of the monomer.

Results

One of the best fit peaks was deconvoluted to be centered at 23533 Dalton (Figure 3). The deconvoluted mass was consistent with the molecular mass calculated from SDS-PAGE based method. It was also in close approximation with the values reported by earlier investigators that used *Hansenula polymorpha* (Shen *et al*. 1990) or related yeast species as host cell (Wampler *et al*. 1985). Several other low intensity peaks were observed in the range of 24000 Dalton to 30000 Dalton which might represent several glycosylated forms of the protein.

3. Peptide Mapping through Multi-Enzyme Digestion using LC-MS/MS

Methodology

Protein sample was separated over SDS-PAGE gel in three sets. Clearly identified gel-bands were excised, washed and reduced & alkylated using DTT and IAA. The reduced gel-bands were than incubated with appropriate ratio of trypsin solution for 1 hour, Asp-N solution for 1 hour and chymotrypsin solution prepared in calcium chloride buffer for 15 hours at 25° Centigrade respectively. Peptides from the gel-bands were extracted by incubating with 0.5% TFA/ACN solution for 60 minutes at 37° Centigrade. The Supernatant was collected and concentrated at room temperature until moderate dryness. Dried peptides were dissolved in 0.1% TFA solution and was directly infused on HPLC/MS system.

Peptide Separation and Identification using Mass-Spectrometry: Obtained peptides were separated using reverse-phase liquid chromatography using 2-hour gradient and eluate was directly infused into ESI-Q-TOF mass-spectrometer. Peptides MS and MS/MS spectra were acquired, and the compiled mass list was searched against the protein sequence and theoretical trypsin/Asp-n and Chymotrypsin protein digest of rHBsAg.

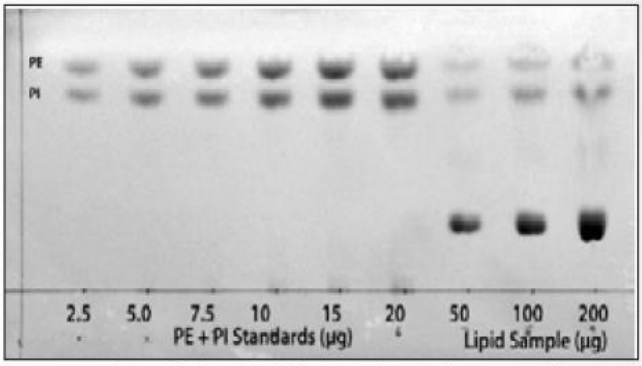

Fig. 8: Analysis of Phosphatidyl Inositol in Lipid extracts of HBsAg.

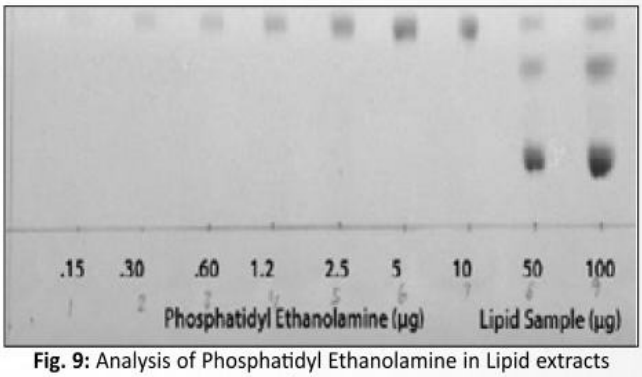

Fig. 9: Analysis of Phosphatidyl Ethanolamine in Lipid extracts of HBsAg.

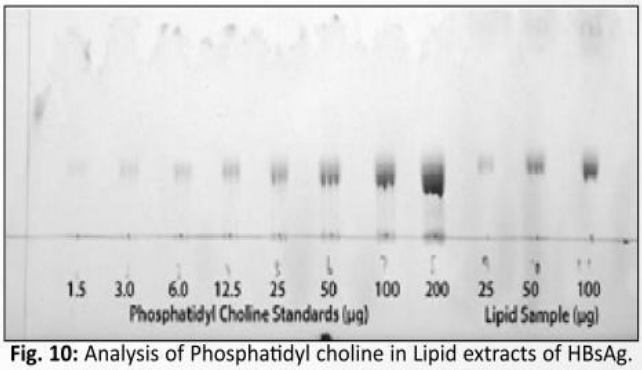

Fig. 10: Analysis of Phosphatidyl choline in Lipid extracts of HBsAg.

Results

A representative Base Pair Chromatogram (BPC) of a protease digest is shown in Figure 4. Presence of several peptide peaks suggest that protein was efficiently digested. The peptide mass search resulted in the 100% amino acid coverage of the known sequence of rHBsAg. This confirmed the identity of the protein to be rHBsAg and confirmed that protein was fully translated. The peptides obtained from the

Neutral Lipids

The neutral lipid contribution in the samples was calculated to be 30.12% of the total extracted lipids. The triglycerides in the sample was around 29.47% of the total extracted lipid while the values of cholesterol and cholesterol ester as obtained through an enzymatic reaction were as shown in (Table 3).

The lipid profile of sample was in agreement with earlier studies (Meeren *et al*. 1994, Gavilanes *et al*. 1982), and it was concluded that Phosphatidyl Choline followed by neutral lipids, are the major lipid species that are present in the HBsAg samples.

8. Characterization of N-linked Glycans

Methodology

To characterize the primary carbohydrate components of HBsAg, N-linked glycans were released from the sample using PNGase F treatment. The obtained glycans were acid hydrolysed, labelled with 8-aminonaphthalene-1,3,6-tri-sulfonic acid (ANTS) and analyzed using Fluorophore Assisted Carbohydrate Electrophoresis (FACE) method (Stack *et al*. 1992, Goins *et al*. 2000). The bands were visualized using gel documentation system under UV light. Images were captured and analyzed by image lab software.

Result

FACE has been successfully used for measurement of relative abundance of Oligosaccharides (Stack *et al*. 1992, Goins *et al*. 2000). The carbohydrate in the sample were identified by comparing the Rf values (electrophoresed distance from origin) of carbohydrate standards. N-acetyl glucosamine and Mannose were found to be the major carbohydrates present in the sample (Figure 9). Other amino sugars and neutral sugars were not detected using this assay. A summary of glycans analyzed, and the results obtained are shown in Table 4.

Study Conclusion

Following conclusions were made in terms of purity, identity and integrity of the sample.

1) Separation of protein on SDS-PAGE established that the protein present in the sample is a multimeric protein. The molecular weight of the monomer of protein was established to be 23.5 kDa.
2) The particle size of the antigen in terms of mean particle diameter was measured as 22.9 nm using differential light scattering method.
3) The identity of the protein sample was established through peptide mapping. The identified peptide sequences over-lapped with 100% of the known protein sequence of HBsAg monomer and provided a confirmatory evidence that the sample is HBsAg antigen.
4) The absence of host cell protein in the sample was confirmed using western-blot analysis and it was established that >99% of the protein component of the sample belong to HBsAg.
5) The lipid to protein ratio in the samples was calculated to be 1.2. Phosphatidyl Choline and Triglycerides constitute was found to be the major components with 63% and 29.47% of the total lipids present in the sample.
6) N-linked glycan analysis concluded that N-acetyl glucosamine (0.67 µg/mg protein) and mannose (2.42 µg/mg protein) are the major carbohydrate species present in the sample.

Shantani's Strength

Shantani has supported physico-chemical characterization of more than 35 different biopharma products. Streamlined characterization workflows that follows ICH-Q6B guidelines under a Quality Management System conferring to ISO 17025:2017, are utilized to provide the best quality services to its collaborators. At the same time a team of dedicated, specialized protein scientists utilizes their decade old expertise in providing, path forwarding solutions for complex biopharma product development problems.

Acknowledgement

We would like to thank the management of Shantani Proteome Analytics Pvt. Ltd., for their support and encouragement. Special thanks to the entire analytical R&D team for their guidance and assistance.

References

1. *Shouval, Daniel., Hepatitis B vaccines Journal of Hepatology, Volume 39, 70 – 76, doi.org/10.1016/S0168-8278(03)00152-1 (2003)*
2. *Bligh et al., A Rapid Method of Total Lipid Extraction and Purification. Canadian Journal of Biochemistry and Physiology, 37:911-917 (1959).*
3. *Gavilanes et al., Structure of Hepatitis B Surface Antigen: Characterization of the Lipid Components and their Association with the Viral Proteins. The Journal of Biological Chemistry, 257:7770-7777, 1982.*
4. *Kee G.S., A rational approach to the development of future generation processes for lipoprotein VLP vaccine candidates. Ph.D. Thesis Submitted to University College London by Kee G.S. 2009.*
5. *Langley et al., Characterization of purified hepatitis B surface antigen containing pre-S (2) epitopes expressed in Saccharomyces cerevisiae. Gene, 67:229-245, 1988.*
6. *Meeren et al., Phospholipid Composition of r-DNA Hepatitis B Surface Antigens. International Journal of Pharmaceutics, 106:89-92, 1994.*
7. *Norder et al., Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hep B virus strains. Journal of General Virology, 73:1201-1208, 1992.*
8. *Shen et al., Excretion of proteins from yeast cells. Patent Publication Number EP0389377 A1, Application Number EP19900400796, Publication Date 26 Sep.1990*
9. *Strods et al., Preparation by alkaline treatment and detailed characterization of empty hepatitis B Virus core particles for vaccine and gene therapy applications. Scientific Reports, 5:11639, doi: 10.1038/srep11639, 2015*
10. *Vnek et al., Heterogeneity of Hepatitis B Surface Antigen-Associated Particles Isolated from Chimpanzee Plasma. Infection and Immunity, 16:335-343, 1977.*
11. *Wampler et al., Multiple chemical forms of hepatitis B surface antigen produced in yeast. Proceedings of the National Academy of Sciences, USA, 82:6830-6834, 1985.*
12. *Zhao et al., Maturation of recombinant hepatitis B virus surface antigen particles. Human Vaccine, 2:174-180, 2006.*