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Determination of Structure of Hepatitis B Virus E Antigen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

Asheel Ketan Patel

Bachelors of Pharmacy, Rajiv Gandhi University of Health Sciences, 2006

Director: Dr. Darrell Peterson, Professor, Department of Biochemistry

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Richmond, Virginia
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Table of Contents

List of Tables	iv
List of Figures	v
Abstract	ix
Introduction	
Hepatitis	1
Hepatitis B Virus	1
Hepatitis B Virus Structure	3
Hepatitis B Virus Genome	6
Background.....	9
Hypothesis	12
Methods and Materials	
Plasmid Construction	14
Expression and Purification	25
ELISA	27
Reduction or Reduction and Alkylation of HBeAg	29
Quantitation of Sulfhydryl groups	30
Circular Dichroism	31
Light Scattering	31
Results and Discussion	
Expression and Purification	32
Evaluation of Disulfide Bonding in HBeAg by 2D SDS PAGE	60
ELISA	62
Reduction or Reduction and Alkylation of HBeAg	72
Quantitation of Sulfhydryl groups	95
Circular Dichroism Spectra of the Recombinant HBeAg and its Mutants	98
Light Scattering	99
Conclusion	108
Future Directions	113
References	114

List of Tables

Table 1. PCR Cycles	22
Table 2. Table showing the total amount of all the proteins obtained	58
Table 3. Spectrophotometer readings at 412nm along with the moles of -SH/ Mole of proteins	96

List of Figures

Figure 1: Hepatitis B Virus geographical distribution	2
Figure 2: The Dane particle	3
Figure 3: The Genome of Hepatitis B Virus	6
Figure 4: Hypothetical disulfide bridging pattern in HBe	12
Figure 5: SDS PAGE Gel picture of wild-type recombinant HBeAg	33
Figure 6: Elution profile for the wild-type recombinant HBeAg protein from Sephacrose CL4B	34
Figure 7: SDS PAGE Gel of fractions obtained from the chromatography of wild- type recombinant HBeAg on Sepharose CL4B	35
Figure 8: SDS PAGE Gel picture of concentrated wild-type recombinant HBeAg	36
Figure 9: SDS PAGE Gel picture of -7S	37
Figure 10: SDS PAGE Gel picture of fractions of -7S	38
Figure 11: SDS PAGE Gel picture of concentrated -7S	39
Figure 12: SDS PAGE Gel picture of -7S A	40
Figure 13: SDS PAGE Gel picture of fractions of -7S A	41
Figure 14: SDS PAGE Gel picture of concentrated -7S A	42
Figure 15: SDS PAGE Gel picture of -7S B	43
Figure 16: SDS PAGE Gel picture of fractions of -7S B	44
Figure 17: SDS PAGE Gel picture of concentrated -7S B	45
Figure 18: SDS PAGE Gel picture of -7S C	46
Figure 19: SDS PAGE Gel picture of fractions of -7S C	47
Figure 20: SDS PAGE Gel picture of concentrated -7S C	48

Figure 21: SDS PAGE Gel picture of PCA	49
Figure 22: SDS PAGE Gel picture of fractions of PCA	50
Figure 23: SDS PAGE Gel picture of concentrated PCA	51
Figure 24: SDS PAGE Gel picture of PCB	52
Figure 25: SDS PAGE Gel picture of fractions of PCB	53
Figure 26: SDS PAGE Gel picture of concentrated PCB	54
Figure 27: SDS PAGE Gel picture of PCC	55
Figure 28: SDS PAGE Gel picture of fractions of PCC	56
Figure 29: SDS PAGE Gel picture of concentrated PCC	57
Figure 30: SDS PAGE Gel showing same concentration of proteins loaded	59
Figure 31: 2-D gel (non-reduced followed by reducing conditions) of recombinant HBeAg	61
Figure 32: Graph showing antigenic activity for wild-type recombinant HBeAg	63
Figure 33: Graph showing antigenic activity for -7S.....	64
Figure 34: Graph showing antigenic activity for -7S A	65
Figure 35: Graph showing antigenic activity for -7S B	66
Figure 36: Graph showing antigenic activity for -7S C	67
Figure 37: Graph showing antigenic activity for PCA	68
Figure 38: Graph showing antigenic activity for PCB	69
Figure 39: Graph showing antigenic activity for PCC	70
Figure 40: Effect of decreasing the concentration of reducing agent on wild-type recombinant HBeAg without iodoacetamide treatment	72
Figure 41: Effect of decreasing the concentration of reducing agent on wild-type recombinant HBeAg with iodoacetamide treatment	74
Figure 42: Inter-molecular disulfide bond formation observed from figure 41	75

Figure 43: Effect of decreasing the concentration of reducing agent on -7S without iodoacetamide treatment	76
Figure 44: Effect of decreasing the concentration of reducing agent on -7S with iodoacetamide treatment	77
Figure 45: Inter-molecular disulfide bond formation observed from figure 44	78
Figure 46: Effect of decreasing the concentration of reducing agent on -7S A without iodoacetamide treatment	79
Figure 47: Effect of decreasing the concentration of reducing agent on -7S A with iodoacetamide. treatment	80
Figure 48: Inter-molecular disulfide bond formation observed from figure 47	81
Figure 49: Effect of decreasing the concentration of reducing agent on -7S B without iodoacetamide treatment	81
Figure 50: Effect of decreasing the concentration of reducing agent on -7S B with iodoacetamide treatment	82
Figure 51: Inter-molecular disulfide bond formation observed from figure 50	83
Figure 52: Effect of decreasing the concentration of reducing agent on -7S C with iodoacetamide treatment	84
Figure 53: Effect of decreasing the concentration of reducing agent on PCC with iodoacetamide treatment	85
Figure 54: Disulfide bond formation observed from figure 53	86
Figure 55: Disulfide bond formation observed from figure 53	86
Figure 56: Effect of decreasing the concentration of reducing agent on PCA without iodoacetamide treatment	88
Figure 57: Effect of decreasing the concentration of reducing agent on PCA with iodoacetamide treatment	89
Figure 58: Inter-molecular disulfide bond formation observed from figure 57	90
Figure 59: Intra-molecular disulfide bond formation observed from figure 57	90
Figure 60: Effect of decreasing the concentration of reducing agent on PCB without iodoacetamide treatment	91

Figure 61: Effect of decreasing the concentration of reducing agent on PCB with iodoacetamide treatment	92
Figure 62: Inter-molecular disulfide bond formation observed from figure 61	93
Figure 63: Intra-molecular disulfide bond formation observed from figure 61	93
Figure 64: Cysteine Standard Curve	95
Figure 65: Circular dichroism of all the proteins	98
Figure 66: Light scatter data showing particle size of wild-type recombinant HBeAg	100
Figure 67: Graph showing 10 data points obtained for wild-type recombinant HBeAg	100
Figure 68: Light scatter data showing particle size of -7S	101
Figure 69: Graph showing 10 data points obtained for -7S	101
Figure 70: Light scatter data showing particle size of -7S A	102
Figure 71: Graph showing 10 data points obtained for -7S A	102
Figure 72: Light scatter data showing particle size of -7S B	103
Figure 73: Graph showing 10 data points obtained for -7S B	103
Figure 74: Light scatter data showing particle size of -7S C	104
Figure 75: Graph showing 10 data points obtained for -7S C	104
Figure 76: Light scatter data showing particle size of PCA	105
Figure 77: Graph showing 10 data points obtained for PCA	105
Figure 78: Light scatter data showing particle size of PCB	106
Figure 79: Graph showing 10 data points obtained for PCB	106
Figure 80: Light scatter data showing particle size of PCC	107
Figure 81: Graph showing 10 data points obtained for PCC	107

Abstract

DETERMINATION OF STRUCTURE OF HEPATITIS B VIRUS E ANTIGEN

By

Asheel Patel, Bachelors in Pharmacy

A thesis submitted in partial fulfillment of the requirements for the degree of Masters

of Science at Virginia Commonwealth University

Major Director: Dr. Darrell Peterson
Professor, Department of Biochemistry

Virginia Commonwealth University, 2010

Hepatitis B virus is a member of the hepadnavirus family. The hepatitis B virus core gene codes for two proteins viz. core protein and pre-core protein. These proteins assemble to form particles viz. HBcAg and HBeAg respectively. The structure of the HBcAg has been widely studied but very little is known about the structure of HBeAg. Therefore, the aim of this study was to identify the disulfide bonding patterns in HBeAg. Recombinant HBeAg was isolated from E.coli and used for this study along with various mutants of HBeAg. There are four cysteines present in HBeAg each at position -7, 48, 61 and 107. From this study it can be inferred that the cysteine at 61 and 48 were found to be involved in inter-molecular disulfide bonds between the cysteine at 61 and 48 of other identical monomers. These di-mers were further inter-molecularly linked with cysteine at -7 to form chains. Moreover, the cysteine at -7 and cysteine at 107 were sometimes involved in intra-molecular disulfide bond formation. Thus, the HBeAg in a solution was found be particulate with a heterogeneous pattern of inter chain disulfide bonds.

Chapter 1: *Introduction*

Hepatitis

Hepatitis is a disease caused by a group of viruses that include Hepatitis A, B, C, D and E. Hepatitis can also be caused by toxins such as alcohol, plants or some medicines. This disease causes inflammation of the liver. The most common viral infections in the United States are hepatitis A, hepatitis B, hepatitis C and hepatitis D. Vaccines for Hepatitis A and B are available but there is no vaccine yet available for hepatitis C. The disease can be acute if it lasts for less than 6 months or chronic if it persists longer than 6 months. The hepatitis A and E cause acute diseases and are transmitted enterically, whereas hepatitis B, C and D viruses may produce chronic diseases and are transmitted via the blood (10). The symptoms of acute viral hepatitis are myalgia, vomiting, nausea, fatigue, a change in the sense of taste and smell, right upper abdominal pain, headache and diarrhoea which may have pale stools or dark urine (11). Chronic hepatitis develops gradually without causing any obvious symptoms in two thirds of people. In the remaining one third, it is caused due to persistent acute hepatitis or because of its return. Symptoms often observed are malaise, fatigue and poor appetite (12).

Hepatitis B Virus (HBV)

Hepatitis B is caused by hepatitis B virus. The hepatitis B virus (HBV) is a member of the hepadnavirus family. There are two types of hepadnaviruses:

- The **mammalian hepadnaviruses** include the human hepatitis B virus, woodchuck hepatitis B virus and ground squirrel hepatitis B virus

- The **avian hepadnaviruses** include the duck hepatitis B virus, heron hepatitis B virus and goose hepatitis B virus

Hepadnaviruses are widespread in nature. Although there are only a few known hepadnaviruses, there are probably many others in nature yet to be discovered.

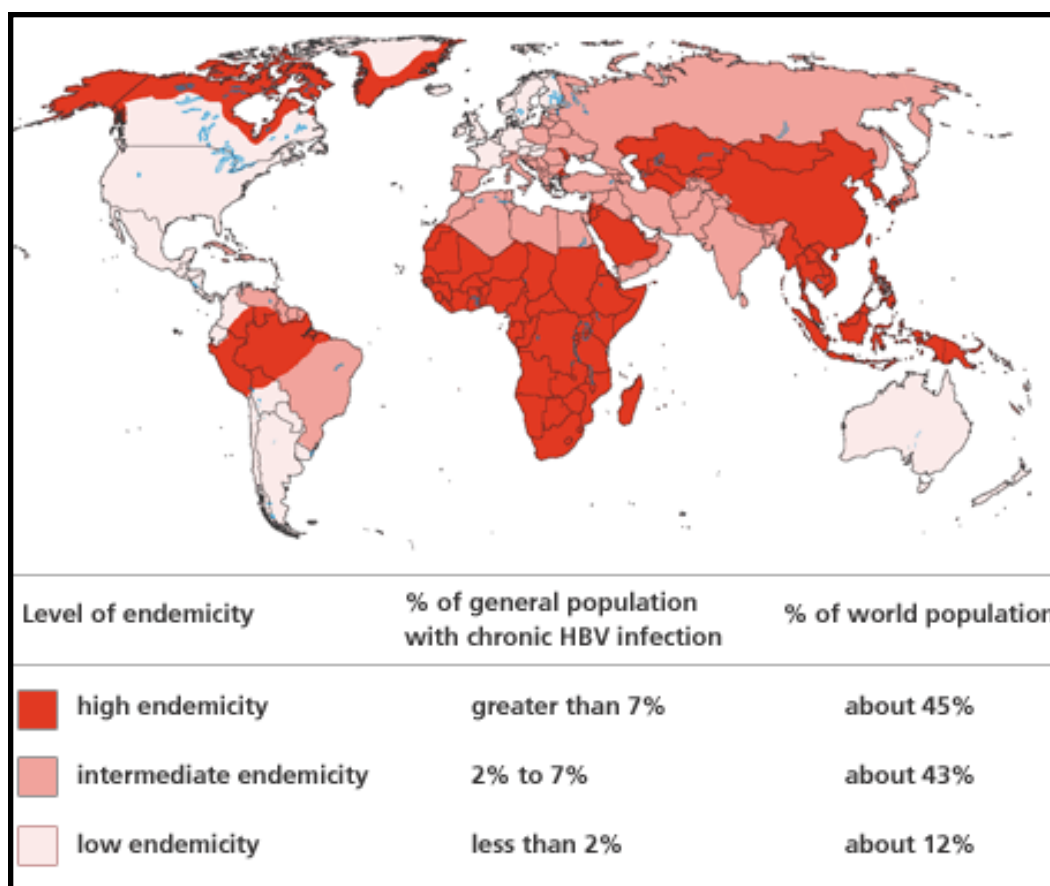


Figure 1. **Hepatitis B Virus geographical distribution (14)**

The hepatitis B Virus is divided into 8 genotypes (A - H). These genotypes have distinct geographical distribution. Moreover, different genotypes cause differences in the severity of the infection and may respond to treatment in different ways. Type A genotype has two subtypes viz. Type A(A1) and type A(A2). The A(A1) subtype is

prevalent in Africa/Asia and Philippines, whereas the A(A2) subtype is prevalent in Europe/United States. The Type B genotype has two subtypes viz. Bj/B1 and Ba/B2. The 'j' for subtype Bj/B1 stands for Japan and the 'a' for subtype Ba/B2 stands for Asia. Moreover, Type C genotype is divided in two subtypes viz. Cs/C1 and Ce/C2. The Cs/C1 subtype is prevalent in South-east Asia whereas the Ce/C2 subtype is prevalent in east Asia. The Type D genotype is present in the Mediterranean area, Middle East and India. It is divided into 7 subtypes (D1 - D7). The Type E genotype is found in sub-Saharan Africa. Type F and Type H are only found in Central and South America. The Type G genotype is present in France and Germany. The genotypes A, D and F are prevalent specifically in Brazil and all the genotypes are present in United States depending on the ethnicity of the area (13).

Hepatitis B Virus Structure

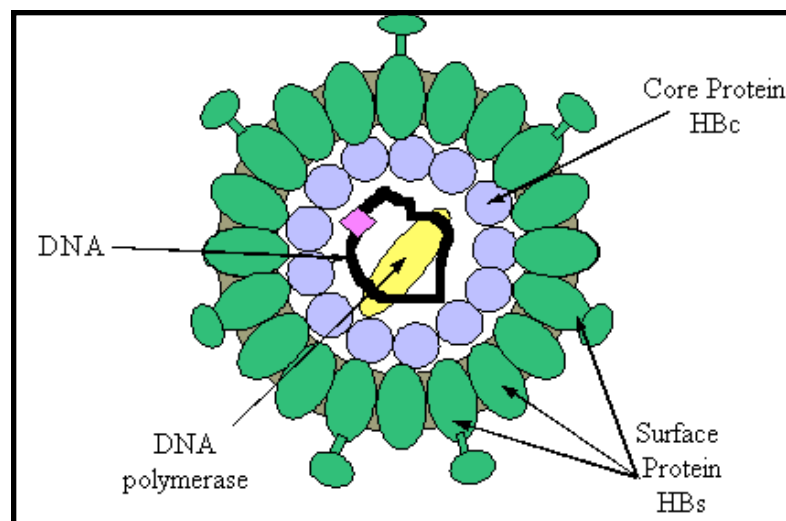


Figure 2. **The Dane particle (15)**

The hepatitis B virus is 42nm in size and is a double-shelled particle that was originally called the Dane particle (15). It consists of a 7nm thick outer shell and a 27nm

inner core. The core contains a DNA polymerase along with a small, circular, partially double-stranded DNA (9).

The hepatitis B virus surface protein (HBs) is associated with complex antigenic determinants present on the surface of HBV. These have a particle size of 22nm and are spherical or tubular in shape (9). The HBc is a major structural component of the HBV viral nucleocapsid and is 27nm in size. The woodchuck hepatitis virus also has a similar protein whereas the duck hepatitis virus has an analogous protein. It has been shown recently that the deletion of 39 amino acids at the carboxyl terminus of the HBc results in the formation of a truncated HBc that still has the capability of forming core particles. However, the truncated HBc no longer had the ability to bind nucleic acids (3, 4, 5).

The pre-core is closely related to the nucleocapsid of HBV. It is a 25 kDa protein. It is N-terminally as well as C-terminally processed and is secreted as non-particulate E antigen (HBeAg). The pre-core protein has extra 29 N-terminal amino acids. These N-terminal amino acids serve as a signal peptide which directs the nascent polypeptide chain into the secretory pathway. The signal sequence is cleaved during translocation into the lumen of the endoplasmic reticulum thus generating a 22 kDa protein. This 22 kDa mature HBeAg is C-terminally truncated at amino acid 149 of HBc and retains only 10 amino acids out of the 29 original pre-core amino acids at the N-terminal, thus generating a 17 kDa protein which is called the HBeAg (6, 16, 17, 18, 19).

The HBeAg was first found by Magnius and Espmark in 1972 (25). It was then shown to be closely associated with the hepatitis B Virus core. The HBeAg was found in sera with the presence of Dane particles (Nordenfelt and Kjellen, 1975 and Takahashi et

al. 1976). It was observed that the HBeAg in serum can be associated with high infectivity (Magnius et al. 1975 and Okada et al. 1976) and it may be a prognostic value in predicting the course of liver disease (Trepo et al, 1976) (20). It was also observed that the HBeAg and the Dane particles had an intimate relationship and the HBeAg demonstrated a signal of high infectivity for transmission of HBV (23). The HBeAg was found to be a homogenous mixture of particles ranging from molecular weight of 200000 to 300000 daltons. It was previously stated that this large molecules were formed because of interaction of the IgG with HBeAg (21, 22). But in 1979 it was characterized as an antigenic protein containing determinant borne out of the core protein of the HBV (26). This was further proven by the generation of HBeAg determinants by proteolytic digestion of HBV core expressed in E.coli (20). It has also been stated that HBeAg is a degradation product of HBcAg and this is the reason why HBeAg and not HBcAg is found in the serum of infected patients. The HBeAg is present with free HBV DNA in plasma as well as liver and thus can be used as an diagnostic marker. The HBV DNA is in free state because the DNA binding region at the C-terminal of HBcAg is not available in HBeAg (20). Moreover, three antigenic determinants were characterized. The single conformational determinant responsible for HBc antigenicity in assembled core and a linear HBe related determinant (HBe1) were both found overlapping near the amino acid 80 region. The second determinant (HBe2) was assigned to location around amino acid 138 but to become antigenic, it required the intra-molecular participation of the sequence between amino acids 10 and 140. It has been postulated that the HBcAg and HBeAg have common basic 3D structure which exposes the HBe1 determinant but they

differ in the two conformational determinant that are introduced (HBc) or is masked (HBe2) in the core (27).

Hepatitis B Virus Genome

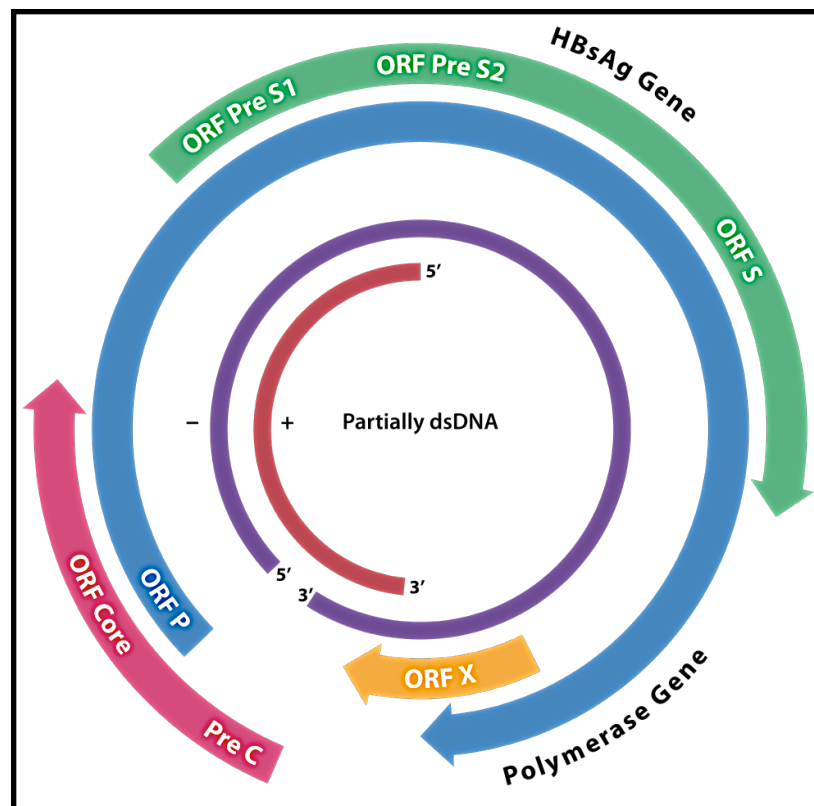


Figure 3. **The Genome of Hepatitis B Virus**

The HBV genome consists of a circular double-stranded DNA. This DNA replicates by a novel reverse transcription process. These viruses have a nucleocapsid which is the major structure of the virus. The nucleocapsid contains genomic DNA, a core structural protein (HBc) and a reverse transcriptase. Moreover, the genome of HBV contains 4 open reading frames (ORFs). Various forms of surface proteins are encoded by the first ORF. Translation is initiated by three in-frame methionine codons and this results in the formation of the Pre S1, Pre S2 and the HBs (1).

The HBV core gene (second ORF) codes for two distinct proteins: a 21.5 kDa protein that assembles to form nucleocapsid particle (HBc) and a pre-core protein that is directed to the endoplasmic reticulum. Therefore, the HBV gene contains two in-phase initiation codons. Translation from the second ATG codon results in the expression of HBc whereas translation from the first ATG codon results in the synthesis of the pre-core protein (1, 5). The third ORF is the largest and it overlaps all the other ORFs. This ORF encodes for the viral DNA polymerase (1). The fourth ORF is named as the 'X' since the function of this gene was not known. It encodes for the HBx which is known to be a transcriptional transactivator. It was later suggested that HBx plays a role in the development of liver cancer by stimulating genes for cell growth and by inactivating growth regulatory molecules (1, 2).

The aim of this project was to study the structure of HBeAg, and specifically to determine the disulfide bonding patterns in HBeAg. Therefore, we are only concerned with HBc and pre-core protein since this amino acid sequence ultimately gives rise to HBeAg.

The following is the sequence of HBcAg:

```

1 mdidpykefg asvellsflp sdffpsirdl ldtasalyrealespehcsp hhtalrqail
61 cwgelmnlat wvg snledpa srelvvsyvn vnmglkirql lwfhisccltf gretvleylv
121 sfgvwirtpp ayrpqnapil stlpettvvr rrgrsprrrt psprrrrsq s prrrrsqsre
181 pqc

```


The following is the sequence of the Pre-core:

```

1  mqlfhlclii sctcptvqas klclgwlwgm didpykefga tvellsflps dffpsvrdll
61 dtasalyrea lespehcsph htalrqailc wgelmtlatw vgnnledpas rdlvvnyvnt
121 nmglkirql1 wfhiscltfg retvleylvs fgvwirtppa yrppnapils tlpettvvrr
181 rrdrgrsrprrrrr tpsrprrrrrrsrq sprrrrrrsrqsrr esrqc

```

The amino acids marked in red, in the above core and pre-core sequences, are not present in the final HBeAg sequence. Moreover, the pre-core sequence has an extra 29 amino acids at the N-terminal of the core protein. These extra 29 amino acids are processed in the endoplasmic reticulum to give the HBeAg. The HBeAg sequence remains with only 10 amino acids at the N-terminal after being processed. These 10 amino acids are marked in blue in the following HBeAg sequence. The molecular weight of HBeAg is 17 kilodaltons.

The following is the sequence of the HBeAg:

```

1  SKLCLGWLWG MDIDPYKEFG ATVELLSFLP SDDFFPSVRDL LDTASALYRE
51  ALESPEHCSP HHTALRQAIL CWGELMTLAT WVGVNLEDPA SRDLVVSyVN
101 TNMGLKFRQL LWFHISCLTF GRETvVIEYLV SFGVWIRTPP AYRPPNAPIL
151 STLPETTVV

```

Chapter 2: *Background*

The structure of HBcAg has been well studied but the structure of the HBeAg is not very well characterized. Even though the core and the pre-core have mostly identical sequences, they have different disulfide bonding patterns. So far, the following research work has been carried out on HBc and HBe.

The disulfide bonding pattern of the hepadnaviral core antigens has been determined by Jian Zheng, Florian Schodel and Darrell Peterson. A set of wild type and mutant human, woodchuck and duck hepatitis viral core proteins were prepared to study the free sulfhydryl groups and also to study disulfide bond formation within the core particles of different species. From their study, they stated that human and woodchuck core proteins contain 4 cysteine residues, whereas the duck core protein contained a single cysteine residue. Moreover, all the viral core proteins assemble into identical core particles and it was observed that disulfide bonds were not essential for core particle formation. They also found that no intra-chain disulfide bonds occurred. Further, Cys107 was a free thiol buried within the particle structure, whereas, Cys48 was present partly as a free sulfhydryl which was exposed at the surface of the particle. Cys61 was always involved in inter-chain disulfide bonds with the identical residues of another monomer whereas, Cys48 was partly involved in making inter-chain disulfide bonds with the identical residues with the same other monomer bound by Cys61. Cys183 was always involved in a disulfide bond with the Cys183 of a different monomer than Cys61 and Cys48. As a result of this disulfide bonding pattern, the HBcAg exists as a highly cross-linked structure that will not even enter a normal SDS polyacrylamide gel under non-

reducing conditions. The woodchuck core protein had the same pattern of bonding, whereas the duck core protein had no disulfide bonds. It had a single free Cys153 which was equivalent to the Cys107 of the mammalian core antigen. This cysteine was buried (5).

Further, Florian Schodel and his group carried out the expression of recombinant HBeAg in *E.coli*. They also isolated C-terminally truncated HBcAg from *E.coli*. They found that HBcAg and truncated HBcAg formed 27nm particles and displayed HBc antigenicity. In contrast, the recombinant HBeAg demonstrated HBe antigenicity but did not form uniform particles. They stated that the presence of the 10 pre-core amino acids prevented the formation of the particle. Therefore, the group carried out tests to analyze which of the amino acids were responsible for preventing the particle formation. They substituted cysteine to glutamine at position 7 and found that the particle forming capabilities were restored. Thus, they concluded that cysteine at position 7 appeared to be responsible for the prevention of particle assembly in the HBeAg biosynthesis pathway and hypothesized that the failure to assemble into uniform particles may be due to additional or different disulfide bonds (6).

Moreover, Georg Wasenauer and his group demonstrated the relevance of cysteine residues for the biosynthesis and antigenicity of human hepatitis B 'E protein'. The group made vaccinia virus recombinants that encoded the HBe protein. They found that the mature form of the secretory core protein (HBe) of human hepatitis B virus contained four cysteines which were located at positions -7, 48, 61 and 107. Also, there was a cysteine at position 183 which was cleaved during maturation of the protein. Further, they also found from their studies that the cysteines at position -7 and 61 were

crucial for HBe biosynthesis. Moreover, the di-mer formation was due to Cys-61-Cys-61 disulfide bridges that were formed only if the cysteine at -7 was not present. In the wild type, these disulfide bridges between two cysteines 61 were inhibited by an intra-molecular disulfide bridge between the cysteine at position -7 and one of the internal cysteines. They further observed that the cysteine at position 61 was important for HBe biosynthesis because on mutating this cysteine, the HBe proteins were formed but were either poorly secreted or possessed different antigenicity (7).

Michael Nassal and his group found that an intra-molecular disulfide bridge between Cys-7 and Cys61 determined the structure of the secretory core gene product HBeAg of hepatitis B virus which were obtained from human liver cell lines. They also found that Cys-7 formed an intra-molecular disulfide bond to Cys61. In the absence of Cys-7, secretion was relatively efficient and independent of Cys61. However, the molecules were exported as homodimers exhibiting both HBe and HBc antigenicity. In the absence of Cys61, the non-paired Cys-7 interfered with secretion efficiency. Moreover, they also observed that the amino acids flanking Cys-7 also contributed to the formation of disulfide bonds. They concluded that the Cys-7/Cys61 bond imposed a conformational change that helped in the secretion of the protein and also gave the protein distinct biophysical and antigenic properties (8).

Chapter 3: *Hypothesis*

HBcAg and HBeAg share a large portion of their amino acid sequence. However, HBcAg assembles into uniform particles, while HBeAg has been reported to be heterogeneous in size. Because the truncated HBcAg and full length HBcAg have identical disulfide bonding patterns, and identical particle morphology, and because HBeAg differs from the truncated HBcAg only by the presence of the 10 amino acid pre-core sequence, which contains an additional cysteine, we hypothesize that differences in disulfide bonding may contribute to their structural and antigenic differences. An inter-molecular disulfide bond formed between Cys61 of identical monomers gives rise to the di-mers which form HBeAg. We hypothesize that either this structure cannot form or if it does still form in HBeAg, its further assembly into normal particulate HBeAg is prevented by disulfide bonds involving Cys-7 to form a chain. The following is the diagrammatic representation of the hypothesis.

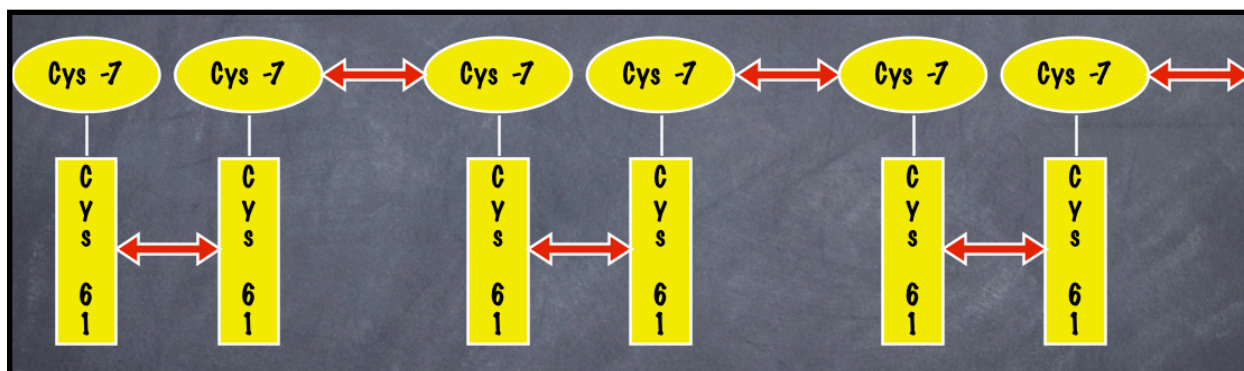


Figure 4. **Hypothetical disulfide bridging pattern in HBe**

In order to test the hypothesis, the following experiments were carried out on native and mutant recombinant HBeAg:

1. Expression and purification of recombinant HBeAg in E.coli.
2. Expression and purification of mutants of HBeAg with serine substitutions at cysteine residues.
3. Circular dichroism, Light scattering, Estimation of free sulfhydryl groups using Ellman's reagent and Effect of decreasing concentration of reducing agent on HBeAg and its mutants to distinguish structural characteristics and disulfide bonding pattern.

Chapter 4: *Methods and Materials*

Plasmid Construction

Dr. Darrell Peterson constructed the HBeAg and the -7S plasmids. The vector used to construct the plasmids was pKK223-3. The following are the plasmids and their mutations which were synthesized using the HBeAg and -7S plasmids as template DNA:

1. **HBeAg**: Wild type
2. **-7S**: HBe with mutation of cysteine at position -7 to a serine
3. **-7S A**: HBe with mutation of cysteine at position -7 and 48 to a serine
4. **-7S B**: HBe with mutation of cysteine at position -7 and 61 to a serine
5. **-7S C**: HBe with mutation of cysteine at position -7 and 107 to a serine
6. **PCA**: HBe with mutation of cysteine at position 48 to a serine
7. **PCB**: HBe with mutation of cysteine at position 61 to a serine
8. **PCC**: HBe with mutation of cysteine at position 107 to a serine

The Stratagene Quickchangel II Site-directed Mutagenesis kit was used to carry out the mutations. The first step was to design primers and order their synthesis. The following parameters were used to design primers:

- All primers were 25 - 45 base pairs in length.
- The melting point of all the primers was not more than 78 degrees C.

- The primers were designed in such a way that the desired mutation was in the middle of the primer and there were 10 - 15 base pairs flanking on each side of the mutation.

- Moreover, the minimum GC content was 40% and all the primers terminated with one or more G or C.

Once the primers were designed, they were synthesized by the VCU Nucleic Acids Core Facility. The primers, along with the appropriate template were then used to make the mutants. For mutants PCA, PCB and PCC, HBeAg plasmid was used as the template DNA since only the cysteine at positions 48, 61 and 107 respectively were supposed to be mutated. For mutants -7S A, -7S B and -7S C, the -7S plasmid was used as the template DNA, since it already had the cysteine at -7 mutated and so further only mutations of cysteine at positions 48, 61 and 107 respectively had to be carried out.

The following are the primers synthesized:

1. TTA GAG TCT CCT GAG CAT TCT TCA CCT CAC CAT ACT GCA CTC
2. GAG TGC AGT ATG GTG AGG TGA AGA ATG CTC AGG AGA CTC TAA
3. AGG CAA GCA ATT CTT TCC TGG GGG GAA CTA ATG
4. CAT TAG TTC CCC CCA GGA AAG AAT TGC TTG CCT
5. CTC TTG TGG TTT CAC ATT TCT TCT CTC ACT TTT GGA AGA G
6. C TCT TCC AAA AGT GAG AGA AGA AAT GTG AAA CCA CAA GAG

The following are the sequences of the template DNA with the primers for each mutant shown at the position of each mutation.

PCA

```

ttgtgagcggataaacaattttcacataaggaggaaaaaaccATGGATATCGAtCCTTATAA
1  -----+-----+-----+-----+-----+-----+ 60
aacactcgccctattgttaaagtgtattcctccttttttggTACCTATAGCTaGGAATATT

a   L * A D N N F T * G G K N H G Y R S L * -
b   C E R I T I S H K E E K T M D I D P Y K -
c   V S G * Q F H I R R K K P W I S I L I K -

AGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTCTTCAGT
61  -----+-----+-----+-----+-----+-----+ 120
TCTTAAACCTCGATGACACCTCAATGAGAGCAAAAACGGAAGACTGAAGAAAGGAAGTCA

a   R I W S Y C G V T L V F A F * L L S F S -
b   E F G A T V E L L S F L P S D F F P S V -
c   N L E L L W S Y S R F C L L T S F L Q Y -

BgIII
|
ACGAGATCTTCTAGATAACCGCCTCAGCTCTGTATCGGGAAGCCTTAGAGTCTCCTGAGCA
121 -----+-----+-----+-----+-----+-----+ 180
TGCTCTAGAAGATCTATGGCGGAGTCGAGACATAGCCCTTCGGAATCTCAGAGGACTCGT

a   T R S S R Y R L S S V S G S L R V S * A -
b   R D L L D T A S A L Y R E A L E S P E H -
c   E I F * I P P Q L C I G K P * S L L S I -

TTCTTCACCTCACCATACTGCACTC
TTGTTTCACCTCACCATACTGCACTCAGGCAAGCAATTCTTTGCTGGGGGGAACATAATGAC
181 -----+-----+-----+-----+-----+-----+ 240
AACAAGTGGAGTGGTATGACGTGAGTCCGTTTCGTTAAGAAACGACCCCCCTTGATTACTG

a   L F T S P Y C T Q A S N S L L G G T N D -
b   C S P H H T A L R Q A I L C W G E L M T -
c   V H L T I L H S G K Q F F A G G N * * L -

TCTAGCTACCTGGGTGGGTGTTAATTTGGAAGATCCAGCGTCTAGAGACCTAGTAGTCAG
241 -----+-----+-----+-----+-----+-----+ 300
AGATCGATGGACCCACCCACAATTAAACCTTCTAGGTGCGAGATCTCTGGATCATCAGTC

a   S S Y L G G C * F G R S S V * R P S S Q -
b   L A T W V G V N L E D P A S R D L V V S -
c   * L P G W V L I W K I Q R L E T * * S V -

TTATGTCAACACTAATATGGGCCTAAAGTTCAGGCAACTCTTGTGGTTTTACATTTCTTG
301 -----+-----+-----+-----+-----+-----+ 360
AATACAGTTGTGATTATAACCCGGATTTCAAGTCCGTTGAGAACACCAAAGTGTAAGAAC

a   L C Q H * Y G P K V Q A T L V V S H F L -
b   Y V N T N M G L K F R Q L L W F H I S C -
c   M S T L I W A * S S G N S C G F T F L V -

TCTCACTTTTGGGAAGAGAAACAGTTATAGAGTATTTGGTGTCTTTTCGGAGTGTGGATTTCG
361 -----+-----+-----+-----+-----+-----+ 420
AGAGTGAAAACCTTCTCTTTGTCAATATCTCATAAACCACAGAAAGCCTCACACCTAAGC

a   S H F W K R N S Y R V F G V F R S V D S -

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b      L T F G R E T V I E Y L V S F G V W I R -
c      S L L E E K Q L * S I W C L S E C G F A -

      CACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCCTATCAACACTTCCGGAGACTAC
421 -----+-----+-----+-----+-----+-----+ 480
      GTGAGGAGGTCTGAATATCTGGTGGTTTACGGGGATAGGATAGTTGTGAAGGCCTCTGATG

a      H S S S L * T T K C P Y P I N T S G D Y -
b      T P P A Y R P P N A P I L S T L P E T T -
c      L L Q L I D H Q M P L S Y Q H F R R L L -

      TGTGTGTTAGACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG
481 -----+-----+-----+-----+-----+-----+ 540
      ACAACAATCTGCTGCTCCGTCCAGGGGATCTTCTTCTTGAGGGAGCGGAGCGTCTGCTTC

a      C C * T T R Q V P * K K N S L A S Q T K -
b      V V R R R G R S P R R R T P S P R R R R -
c      L L D D E A G P L E E E L P R L A D E G -

                        BglII
                        |
      GTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAGTATTCCTT
541 -----+-----+-----+-----+-----+-----+ 600
      CAGAGTTAGCGGCGCAGCGTCTTCTAGAGTTAGAGCCCTTAGAGTTACAATCATAAGGAA

a      V S I A A S Q K I S I S G I S M L V F L -
b      S Q S P R R R R S Q S R E S Q C * Y S L -
c      L N R R V A E D L N L G N L N V S I P W -

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Forward Primer: TTA GAG TCT CCT GAG CAT TCT TCA CCT CAC CAT ACT GCA CTC

Reverse Primer: GAG TGC AGT ATG GTG AGG TGAAGAATG CTC AGG AGA CTC TAA

Length: 42

GC Content: 47.6 %

Melting Temp.: 66.1 °C

PCB

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ttgtgagcggataacaattttcacataaggaggaaaaaaccATGGATATCGAtCCTTATAA
1  -----+-----+-----+-----+-----+-----+ 60
aacactcgcctattgttaaagtgtattcctccttttttggTACCTATAGCTaGGAATATT

a      L * A D N N F T * G G K N H G Y R S L * -
b      C E R I T I S H K E E K T M D I D P Y K -
c      V S G * Q F H I R R K K P W I S I L I K -

AGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTTCCTTCAGT
61  -----+-----+-----+-----+-----+-----+ 120
TCTTAAACCTCGATGACACCTCAATGAGAGCAAAAACGGAAGACTGAAGAAAGGAAGTCA

a      R I W S Y C G V T L V F A F * L L S F S -
b      E F G A T V E L L S F L P S D F F P S V -
c      N L E L L W S Y S R F C L L T S F L Q Y -

Bg1II
|
ACGAGATCTTCTAGATACCGCCTCAGCTCTGTATCGGGAAGCCTTAGAGTCTCCTGAGCA
121 -----+-----+-----+-----+-----+-----+ 180
TGCTCTAGAAGATCTATGGCGGAGTCGAGACATAGCCCTTCGGAATCTCAGAGGACTCGT

a      T R S S R Y R L S S V S G S L R V S * A -
b      R D L L D T A S A L Y R E A L E S P E H -
c      E I F * I P P Q L C I G K P * S L L S I -

                                AGGCAAGCAATTCTTTTCCTGGGGGGAAC TAATG
TTGTTACCTCACCATACTGCACTCAGGCAAGCAATTCTTTGCTGGGGGGAAC TAATGAC
181 -----+-----+-----+-----+-----+-----+ 240
AACAAGTGGAGTGGTATGACGTGAGTCCGTTTCGTTAAGAAACGACCCCCCTTGATTACTG

a      L F T S P Y C T Q A S N S L L G G T N D -
b      C S P H H T A L R Q A I L C W G E L M T -
c      V H L T I L H S G K Q F F A G G N * * L -

TCTAGCTACCTGGGTGGGTGTTAATTTGGAAGATCCAGCGTCTAGAGACCTAGTAGTCAG
241 -----+-----+-----+-----+-----+-----+ 300
AGATCGATGGACCCACCCACAATTAACCTTCTAGGTGCGAGATCTCTGGATCATCAGTC

a      S S Y L G G C * F G R S S V * R P S S Q -
b      L A T W V G V N L E D P A S R D L V V S -
c      * L P G W V L I W K I Q R L E T * * S V -

TTATGTCAACACTAATATGGGCCTAAAGTTTCAGGCAACTCTTGTGGTTTTACATTTCTTG
301 -----+-----+-----+-----+-----+-----+ 360
AATACAGTTGTGATTATACCCGATTTCAGTCCGTTGAGAACACCAAAGTGTAAGAAGC

a      L C Q H * Y G P K V Q A T L V V S H F L -
b      Y V N T N M G L K F R Q L L W F H I S C -
c      M S T L I W A * S S G N S C G F T F L V -

TCTCACTTTTGGGAAGAGAAACAGTTATAGAGTATTTGGTGTCTTTTCGGAGTGTGGATTGCG

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361 -----+-----+-----+-----+-----+-----+ 420
AGAGTGAAAACCTTCTCTTTGTCAATATCTCATAAACACAGAAAGCCTCACACCTAAGC

a   S H F W K R N S Y R V F G V F R S V D S -
b   L T F G R E T V I E Y L V S F G V W I R -
c   S L L E E K Q L * S I W C L S E C G F A -

CACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCCTATCAACACTTCCGGAGACTAC
421 -----+-----+-----+-----+-----+-----+ 480
GTGAGGAGGTCTGAATATCTGGTGGTTTACGGGGATAGGATAGTTGTGAAGGCCTCTGATG

a   H S S S L * T T K C P Y P I N T S G D Y -
b   T P P A Y R P P N A P I L S T L P E T T -
c   L L Q L I D H Q M P L S Y Q H F R R L L -

TGTTGTTAGACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG
481 -----+-----+-----+-----+-----+-----+ 540
ACAACAATCTGCTGCTCCGTCCAGGGGATCTTCTTCTTGAGGGAGCGGAGCGTCTGCTTC

a   C C * T T R Q V P * K K N S L A S Q T K -
b   V V R R R G R S P R R R T P S P R R R R -
c   L L D D E A G P L E E E L P R L A D E G -

                                BglII
                                |
GTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAGTATTCCTT
541 -----+-----+-----+-----+-----+-----+ 600
CAGAGTTAGCGGCGCAGCGTCTTCTAGAGTTAGAGCCCTTAGAGTTACAATCATAAGGAA

a   V S I A A S Q K I S I S G I S M L V F L -
b   S Q S P R R R R S Q S R E S Q C * Y S L -
c   L N R R V A E D L N L G N L N V S I P W -

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Forward Primer: AGG CAA GCA ATT CTT TCC TGG GGG GAA CTA ATG

Reverse Primer: CAT TAG TTC CCC CCA GGAAAG AAT TGC TTG CCT

Length: 33

GC Content: 48.5 %

Melting Temp.: 65.0 °C

PCC

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ttgtgagcggataacaatttcacataaggaggaaaaaaccATGGATATCGAtCCTTATAA
1  -----+-----+-----+-----+-----+-----+ 60
aacactcgcctattgttaaagtgtattcctccttttttggTACCTATAGCTaGGAATATT

a      L * A D N N F T * G G K N H G Y R S L * -
b      C E R I T I S H K E E K T M D I D P Y K -
c      V S G * Q F H I R R K K P W I S I L I K -

AGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTTCCTTCAGT
61  -----+-----+-----+-----+-----+-----+ 120
TCTTAAACCTCGATGACACCTCAATGAGAGCAAAAACGGAAGACTGAAGAAAGGAAGTCA

a      R I W S Y C G V T L V F A F * L L S F S -
b      E F G A T V E L L S F L P S D F F P S V -
c      N L E L L W S Y S R F C L L T S F L Q Y -

Bg1II
|
ACGAGATCTTCTAGATACCGCCTCAGCTCTGTATCGGGAAGCCTTAGAGTCTCCTGAGCA
121 -----+-----+-----+-----+-----+-----+ 180
TGCTCTAGAAGATCTATGGCGGAGTCGAGACATAGCCCTTCGGAATCTCAGAGGACTCGT

a      T R S S R Y R L S S V S G S L R V S * A -
b      R D L L D T A S A L Y R E A L E S P E H -
c      E I F * I P P Q L C I G K P * S L L S I -

TTGTTACCTCACCATACTGCACTCAGGCAAGCAATTCTTTGCTGGGGGGAACATAATGAC
181 -----+-----+-----+-----+-----+-----+ 240
AACAAGTGGAGTGGTATGACGTGAGTCCGTTTCGTTAAGAAACGACCCCCCTTGATTACTG

a      L F T S P Y C T Q A S N S L L G G T N D -
b      C S P H H T A L R Q A I L C W G E L M T -
c      V H L T I L H S G K Q F F A G G N * * L -

TCTAGCTACCTGGGTGGGTGTTAATTTGGAAGATCCAGCGTCTAGAGACCTAGTAGTCAG
241 -----+-----+-----+-----+-----+-----+ 300
AGATCGATGGACCCACCCACAATTAAACCTTCTAGGTGCGAGATCTCTGGATCATCAGTC

a      S S Y L G G C * F G R S S V * R P S S Q -
b      L A T W V G V N L E D P A S R D L V V S -
c      * L P G W V L I W K I Q R L E T * * S V -

CTCTTGTTGGTTTCACATTTCTTC
TTATGTCAACACTAATATGGGCCTAAAGTTTCAGGCAACTCTTGTGGTTTCACATTTCTTG
301 -----+-----+-----+-----+-----+-----+ 360
AATACAGTTGTGATTATACCCGGATTTCAAGTCCGTTGAGAACACCAAAGTGTAAGAAC

a      L C Q H * Y G P K V Q A T L V V S H F L -
b      Y V N T N M G L K F R Q L L W F H I S C -
c      M S T L I W A * S S G N S C G F T F L V -

TCTCACTTTTGGGAAGAG
TCTCACTTTTGGGAAGAGAACAGTTATAGAGTATTTGGTGTCTTTCGGAGTGTGGATTGCG

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361 -----+-----+-----+-----+-----+ 420
AGAGTGAAAACCTTCTCTTTGTCAATATCTCATAAACACAGAAAGCCTCACACCTAAGC

a   S   H   F   W   K   R   N   S   Y   R   V   F   G   V   F   R   S   V   D   S   -
b   L   T   F   G   R   E   T   V   I   E   Y   L   V   S   F   G   V   W   I   R   -
c   S   L   L   E   E   K   Q   L   *   S   I   W   C   L   S   E   C   G   F   A   -

CACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCCTATCAACACTTCCGGAGACTAC
421 -----+-----+-----+-----+-----+ 480
GTGAGGAGGTCTGAATATCTGGTGGTTTACGGGGATAGGATAGTTGTGAAGGCCTCTGATG

a   H   S   S   S   L   *   T   T   K   C   P   Y   P   I   N   T   S   G   D   Y   -
b   T   P   P   A   Y   R   P   P   N   A   P   I   L   S   T   L   P   E   T   T   -
c   L   L   Q   L   I   D   H   Q   M   P   L   S   Y   Q   H   F   R   R   L   L   -

TGTTGTTAGACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG
481 -----+-----+-----+-----+-----+ 540
ACAACAATCTGCTGCTCCGTCCAGGGGATCTTCTTCTTGAGGGAGCGGAGCGTCTGCTTC

a   C   C   *   T   T   R   Q   V   P   *   K   K   N   S   L   A   S   Q   T   K   -
b   V   V   R   R   R   G   R   S   P   R   R   R   T   P   S   P   R   R   R   R   -
c   L   L   D   D   E   A   G   P   L   E   E   E   L   P   R   L   A   D   E   G   -

                                BglII
                                |
GTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGAATCTCAATGTTAGTATTCCTT
541 -----+-----+-----+-----+-----+ 600
CAGAGTTAGCGGCGCAGCGTCTTCTAGAGTTAGAGCCCTTAGAGTTACAATCATAAGGAA

a   V   S   I   A   A   S   Q   K   I   S   I   S   G   I   S   M   L   V   F   L   -
b   S   Q   S   P   R   R   R   R   S   Q   S   R   E   S   Q   C   *   Y   S   L   -
c   L   N   R   R   V   A   E   D   L   N   L   G   N   L   N   V   S   I   P   W   -

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Forward Primer: CTC TTG TGG TTT CAC ATT TCT TCT CTC ACT TTT GGA AGA G

Reverse Primer: C TCT TCC AAA AGT GAG AGA AGA AAT GTG AAA CCA CAA GAG

Length: 40

GC Content: 40.0 %

Melting Temp.: 62.2 °C

The same above mentioned forward and reverse primers were used for synthesis of mutants -7S A, -7S B and -7S C since the same point mutations were to be carried out except that these mutants also had the cysteine at position -7 mutated. In these cases, the DNA template used was the -7S plasmid since this mutant already had the cysteine at -7 mutated.

Mutations were carried out by PCR, according to the directions supplied in the Quick Change kit. The reaction mixtures for the PCR reactions contained: 5µl of 10X reaction buffer, 3µl of template plasmid, 1µl of the appropriate mutagenic primer, 1µl of dNTP mix, and 39µl of water to give a final volume of 50µl.

To all of the above reaction mixtures, 1µl of *pfu*DNA polymerase was added. The reaction mixtures were then put in a PCR machine and the following program was set:

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	16	95°C	30 seconds
		55°C	1 minute
		68°C	5 minutes

Table 1. **PCR Cycles**

After the PCR reaction was carried out, the tubes were kept on ice for 2 minutes so that the reaction was cooled.

The PCR products were then subjected to digestion with *Dpn* I restriction enzyme in order to digest the non-mutated, methylated parental DNA strand. To do this, 1 µl of *Dpn* I restriction enzyme was added to each of the PCR products. The PCR products were mixed and then incubated at 37 °C for 1 hour.

These *Dpn* I treated PCR products were transformed in XL1-Blue Supercompetent cells. The XL1-Blue cells were thawed and 50µl of cells were taken to transform each PCR product. To this 1µl of *Dpn* I treated PCR product was added. This mixture was mixed and was incubated on ice for 30 minutes. The mixture was then heat

pulsed at 42°C for 45 seconds. Then the mixture was immediately put on ice for 2 minutes. To this mixture, 500µl of LB was added and it was incubated at 37°C for 1 hour.

This mixture was then plated on ampicillin plates and incubated for 37°C for more than 16 hours. After 16 hours, the plates had colonies. Colonies from each plate were picked and inoculated in 10ml of LB containing 10µl of ampicillin (50µg/ml) and incubated at 37°C in a shaker overnight.

Plasmid purification was then carried out on all the 10ml cultures by using the Qiagen QIAprep Spin protocol, exactly according to the directions supplied by the company.

Thus, the plasmids of all the mutants were obtained this way and they were sent for sequencing at the DNA sequencing core facility. The following are the sequences of the proteins that would be synthesized from each plasmid as determined by translation of the sequence obtained. In the following sequences the mutations are marked in red.

-7S A

Reading Frame 2 – Forward Primer

VRDLLDTASALYREAL**ESPEH****SPHHTALRQAILCWGELMTLATWVGVNLEDP**
ASRDLVVS**YVNTNMGLKFRQLLWFHISCLTFGRET****VIEYLV****SFGVWIRTPPAYRPPNAP**
IL

Reading Frame 3 - Reverse Primer

MSKL**SLGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAL**
ESPEH**SPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVS****YVNTNMGLKFR**
QLLWFHISCLTFGRET**VIEYLV****SFGV**

-7S B

Reading Frame 1 – Forward Primer

VRDLLDTASALYREAL**ESPEHCSPHHTALRQAIL****S****WGELMTLATWVGVNLED****P**
ASRDLVVS**YVNTNMGLKFR****Q****LLWFHISCLTFGRET****VIEYLV****SFGVWIRTPPAYRPPNAP**
ILSTLPETTVV

Reading Frame 3 – Reverse Primer

MSKL**S****LGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAL**
ESPEHCSPHHTALRQAIL**S****WGELMTLATWVGVNLED****PASRDLVVS****YVNTNMGLKFR**
Q**LLWFHISCLTFGRET****VIEYLV****SFGV**

-7S C

Reading Frame 1 - Reverse Primer

MSKL**S****LGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAL**
ESPEHCSPHHTALRQAILC**WGELMTLATWVGVNLED****PASRDLVVS****YVNTNMGLKFR**
Q**LLWFHIS****S****LT****FGRET****VIEYLV****SFGV**

PCA

Reading frame 2 - Reverse Primer

MSKLCL**GW****LWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAL**
ESPEH**S****PHHTALRQAILC****WGELMTLATWVGVNLED****PASRDLVVS****YVNTNMGLKFR**
Q**LLWFHISCLTFGRET****VIEYLV****SFGV**

PCB

Reading Frame 2 - Reverse Primer

**MSKLCLGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAL
ESPEHCSPHHTALRQAIL^SWGELMTLATWVGVNLEDPASRDLVVS YVNTNMGLKFR
QLLWFHISCLTFGRET VIEYLV SFGV**

PCC

Reading Frame 1 - Reverse Primer

**MSKLCLGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAL
ESPEHCSPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVS YVNTNMGLKFR
QLLWFHIS^SLTFGRET VIEYLV SFGV**

Expression and Purification

The plasmids for all the mutants were freshly transformed into E.coli every time before expression. This was because it was observed that after repeated passage, the bacteria exhibited a large decrease in the level of protein expression. Further, it was also observed that when some of the mutants when transformed in different strains of E.coli competent cells (top10 or TB1), the level of protein expression was higher as measured by higher antigenic activity. Therefore, the HBeAg, PCA, PCB, PCC and -7S C plasmids were transformed into E.coli TB1 competent cells whereas, -7S, -7S A and -7S B plasmids were transformed into E.coli Top 10 competent cells.

The transformation was carried out via electroporation. For this 2mm electroporation cuvettes were used. 50µl of electro-competent TB1/Top 10 cells were

taken and 1.5µl of plasmid was added to it. This was mixed well and it was allowed to sit on ice for 15 minutes. The mixture was then transferred to the 2mm electroporation cuvette and put in the electroporator. The mixture was pulsed and immediately taken out from the electroporation cuvette, 1ml of LB was added to it. This was then incubated on a shaker at 37°C for 1 hour. After an hour, 20µl from it was plated on ampicillin plates and these plates were incubated at 37°C for 16 hours.

A colony was picked up from the plate and inoculated in 10ml of LB containing 10µl of ampicillin (from ampicillin stock - 50mg/ml). This was allowed to grow on a shaker at 37°C overnight. TYN media was prepared and autoclaved. The following is the recipe for TYN media:

- 100g NZamine
- 100g Yeast Extract
- 50g Sodium Chloride
- Made up to 10 liter with deionized water
- pH was adjusted to 7.5

1 liter of the above was placed in 2 liter flasks and autoclaved. Also, 20% glucose solution (0.2 grams/ml in water) was prepared and autoclaved separately. When cooled, the final TYN medium was prepared by adding 5 ml of the 20 % glucose solution and 1 ml of ampicillin (50 mg/ml) to each liter. 2-3 ml of overnight culture was used to inoculate each flask.

This cultures were incubated at 37°C on a shaker for 24 hours. The cells were then centrifuged at 5000rpm for 15 minutes and the bacterial pellets were collected. These pellets were resuspended in 20mM Tris buffer pH 8. The cells were lysed by

passing them through a high pressure homogenizer (Avestin Emulsiflex) at 20,000 psi pressure. The lysate was then centrifuged at 15000 rpm for 30 minutes and the supernatant was collected. The supernatant was then subjected to Ammonium sulfate (20% saturation) precipitation. It was allowed to stand at 4°C for an hour and then centrifuged at 15000rpm for 30 minutes. The pellets were collected and re-dissolved in a minimum volume of 20mM Tris buffer pH 8.0. These re-dissolved pellets were then fractionated by chromatography on a Cross-linked Sepharose CL4B column. The fractions were collected and the protein elution profile was determined by monitoring the absorbance at 280 nm. Fractions were analyzed by SDS PAGE to determine the location of the desired protein. The fractions containing the protein were pooled. This pooled protein was then concentrated in a centrifugal concentrator with a cut-off of 100,000 molecular weight. All of the proteins were purified according to this procedure.

All the proteins were run on SDS-PAGE gels along with a molecular marker. Further, ELISA was also carried out to check the antigenic activity of the proteins obtained.

ELISA

For carrying out an ELISA, IgG was first purified from the serum of a rabbit that had been immunized with truncated HBcAg using DEAE Affi-gel Blue (Bio-Rad, Inc). This was done using DEAE Affi-gel Blue (Bio-Rad, Inc), according to the manufacturers instructions. Once the pure IgG was obtained, it was used to coat plates. The purified IgG was diluted to a concentration of 20µg/ml, in 1X coating buffer (10 mM sodium bicarbonate/10 mM sodium carbonate buffer). 50µl of this was pipetted in each well and

it was allowed to stand at room temperature for 2 hours. Peroxidase conjugated IgG was also prepared. Periodate-activated horseradish peroxidase was prepared and stored in 1 mg aliquots at -80 degrees until use (24). Aliquots of the purified IgG were freeze dried from 1mM bicarbonate buffer. It, too, was stored in 1mg aliquots. One vial of IgG and one vial of activated peroxidase were thawed and mixed. The IgG dissolved immediately. A solution of 1M sodium cyanoborohydride (MW = 62, therefore 62mg/ml) in 10X coating buffer (10 mM sodium carbonate/10 mM sodium bicarbonate) was then prepared. 20 μ l of the 1M sodium cyanoborohydride solution was added to the peroxidase/IgG solution and it was allowed to stand for 2 hours at room temperature after which it was ready to be used. It was then stored in a refrigerator.

For each assay, one strip was taken out from the previously coated IgG plates and washed with water. 100 μ l of BSA (1gram Bovine Serum Albumin in 50ml of 50mM Tris buffer pH 8.0) was added to all the wells. 10 μ l of the sample was added to the last well and this was then serially diluted by transferring 10 μ l to four other wells. This gave a dilutions of 1:10 – 1:10000. The top two wells were left as negative controls. The strips were allowed to stand for 1 hour after which, they were washed with water. To these wells, 50 μ l of peroxidase-conjugated IgG (2 μ l of peroxidase labelled IgG in 1ml of BSA) was added. This was allowed to stand for 30 minutes. These strips were then washed extensively with water and 50 μ l of substrate (BioRad TMBBlue or Abbott Laboratories OPD) was added.

The color was allowed to develop for 10 minutes and the reaction was then stopped by adding 100 μ l of 1M sulphuric acid to each well. The plate was then read at

450 nm (TMBBlue) or 490 nm (OPD). All the readings were then made on a Bio-Tek plate reader.

Reduction or Reduction and Alkylation of HBeAg

Reduction of HBeAg disulfide bonds

The effects of varying the concentration of reducing agent on HBeAg as well as each of the mutants was examined by SDS PAGE. To carry out this experiment, 40 μ l of SDS sample buffer containing no reducing agent was placed in a micro-centrifuge tube. 13 micro-centrifuge tubes were then taken and 20 μ l of SDS sample buffer containing no reducing agent was added to these tubes. Then, to the first tube containing 40 μ l of SDS sample buffer, 2 μ l of β -mercaptoethanol (14.2 M) was added and mixed properly. 20 μ l was pipetted out from this tube and added to the other tube containing 20 μ l of SDS sample buffer and this was mixed properly. The serial dilution was continued to the 13th micro-centrifuge tube giving final concentrations of mercaptoethanol of 710 mM to 0.16 mM. 30 μ l of protein was then added to all the micro-centrifuge tubes and these were boiled and run on an SDS PAGE gel. The samples were then analyzed by SDS polyacrylamide gel electrophoresis.

Reduction and Alkylation of HBeAg disulfide bonds

In order to prevent possible disulfide interchange or reformation during sample preparation, the above procedure was repeated. However following reduction, Iodoacetamide was added (100 mM) to convert all sulfhydryl groups to the carboxamido derivatives.

Quantitation of Sulfhydryl Groups

Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) was used to quantitate free sulfhydryl groups in the recombinant proteins. The reaction was performed as described in the book 'Current Protocols in Molecular Biology' by Frederick M. with some modifications. A series of standards were prepared having concentrations ranging from 10 to 100 μ M cysteine. The total volume of all the tubes was then made up to 500 μ l with 20mM Tris buffer pH 8.0. 50 μ l of Ellman's reagent (4.0mg/ml in ethyl alcohol) was added and the tubes were allowed to stand at room temperature for 15 minutes. Absorbance was then read at a wavelength of 412nm. The values obtained were used to plot a cysteine standard curve. Thereafter, for determination of the cysteine content of the different proteins, samples of each protein were adjusted to 40 μ M concentration. Two tubes containing 500 μ l of each protein were taken. One tube was treated with 0.1% SDS whereas the other tube was untreated. 50 μ l of the Ellman's reagent was added to these tubes and the tubes were allowed to stand for 15 minutes at room temperature. Then the absorbance at 412 nm was measured. The cysteine content for the proteins was extrapolated from the extinction coefficient calculated from the standard curve.

An attempt was also made to reduce the HBeAg using the ReduceIM column from Pierce Chemical Co. This was used to establish whether the disulfide bonds were buried or were surface accessible for reduction. The exact procedure was followed from the manual provided by the company.

Circular Dichroism

Circular dichroism was used to examine the secondary structures of all the proteins. The proteins were diluted to concentrations between 0.2 - 0.4mg/ml. The CD spectra were then obtained using a Olis CD spectrophotometer.

Light Scattering

Dynamic light scattering was used to analyze the size distribution of the particles (in nanometers) in a solution for each of the proteins. These studies were performed with a Dynapro Dynamic Light Scattering instrument (Protein Solutions, Inc).

Chapter 5: *Results & Discussion*

Expression and Purification

The wild-type recombinant HBeAg plasmid was electroporated into TB1 electro-competent cells. A colony was picked up and grown overnight in 10ml LB containing ampicillin. This culture was then used to inoculate 3 liters of TYN media. After 24 hours incubation the cells were spun down and the pellets were resuspended in 20mM Tris buffer. This re-suspended pellet was then lysed in a high pressure homogenizer and the lysate thus obtained was centrifuged. The supernatant was collected and it was subjected to 20% Ammonium sulfate saturation. It was allowed to stand for an hour at 4°C. This was then centrifuged and the pellet and supernatant were collected.

The following is the gel which shows the steps of the purification process. The lysate was loaded along with the the 20% ammonium sulfate pellet and supernatant.

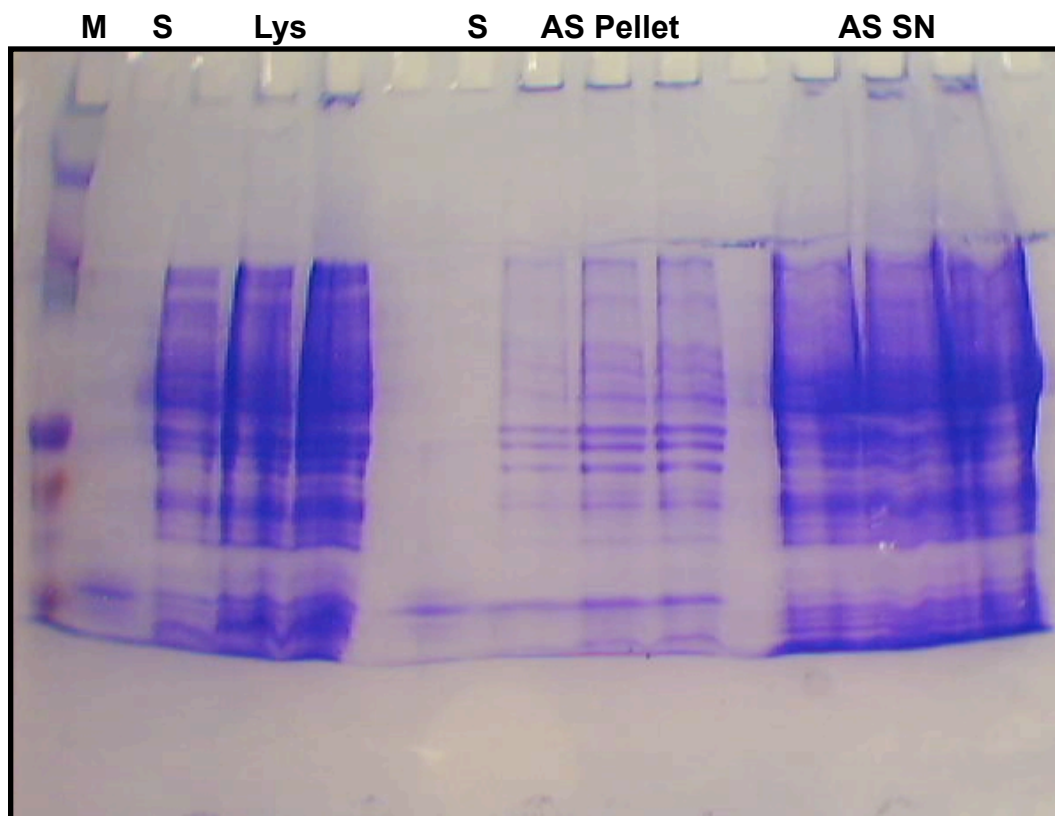


Figure 5. **SDS PAGE gel picture of wild-type recombinant HBeAg.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

Based on the above gel, it is clear that the protein of interest was present in the 20% ammonium sulfate precipitate and this was taken for further purification by gel filtration chromatography.

The 20% ammonium sulfate pellet was re-suspended in 20mM Tris buffer and was loaded on the Sepharose CL4B column. Fractions were collected and the protein elution monitored at 280 nm. The absorbance values obtained for the fractions were plotted on a graph. The following profile was obtained for the wild-type recombinant HBeAg protein. Very similar elution profiles were obtained for all of the proteins. However, the magnitude of the antigen containing peak (as described below) varied.

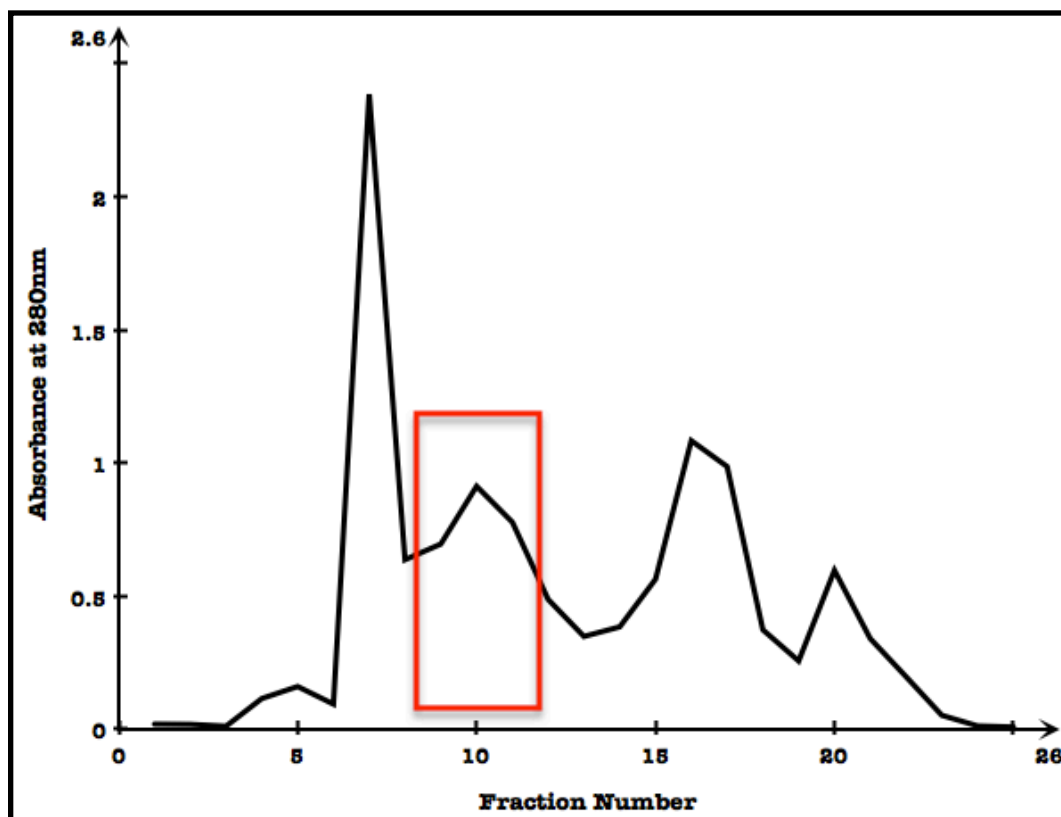


Figure 6. **Elution profile for the wild-type recombinant HBeAg protein from Sepharose CL4B.** The first huge peak contains the 280nm measuring material eluted along with the void volume followed by the second peak which contains the wild-type recombinant HBeAg.

All of the elution profiles had a very similar appearance. A large peak of 280 nm absorbing material eluted at the void volume of the column. Although this peak had high absorbance at 280 nm, it contained little protein that was observable on SDS gels, and thus must represent non-protein components. It was also quite turbid, and much of the absorbance was actually just light scatter. It was in the subsequent fractions that the antigen protein was found, as indicated in the figure above. All the mutants eluted at the same positions. The fractions were run on an SDS PAGE gel to determine which of the fractions contained the wild-type recombinant HBeAg or other antigen protein and to

allow fractions of greatest purity to be pooled. The following is gel picture showing the proteins present in selected fractions.

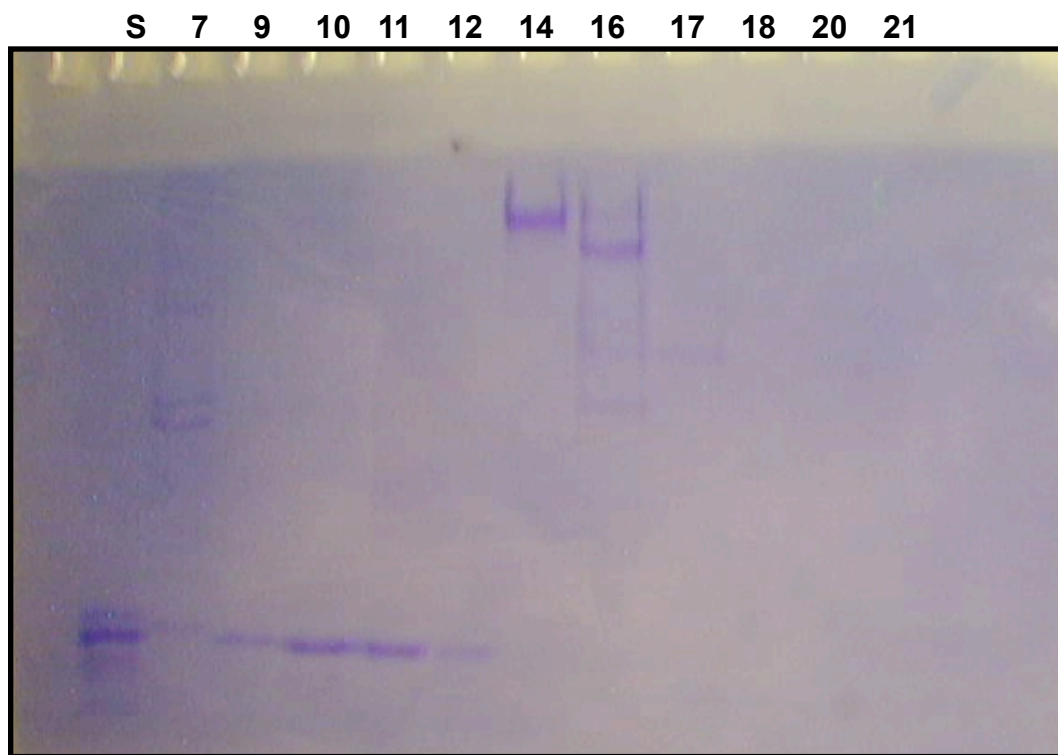


Figure 7. **SDS PAGE Gel of fractions obtained from the chromatography of wild-type recombinant HBeAg on Sepharose CL4B.** Selected fractions from the chromatography were run on the gel.

Fraction 9, 10, 11 and 12 were pooled. The fractions were almost pure. These were then concentrated in a concentrator with a molecular weight cut-off of 100,000. An SDS PAGE gel was run of the top solution and the bottom solution from the concentrator. All of the protein was retained by the concentrator. This was the expected result, since Sepharose CL4B has a molecular weight exclusion limit of about 10 million, the antigen elutes relatively early during the chromatography, it was expected to have an apparent molecular weight of many thousands. It should be noted that all of the

peaks eluting from the Sepharose CL4B column were examined by SDS PAGE, and no additional peaks containing the antigen protein were observed.

The following is the SDS PAGE gel on which the solution retained in the concentrator was run along with the solution at the bottom of the concentrator.

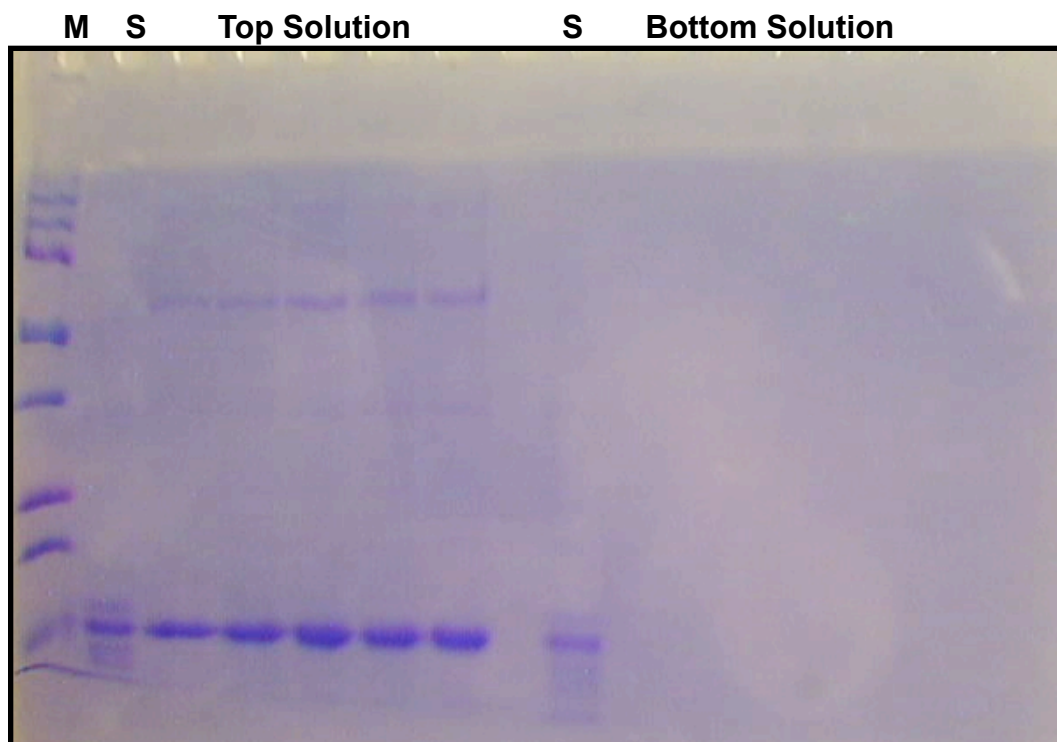


Figure 8. **SDS PAGE Gel picture of concentrated wild-type recombinant HBeAg.** The 'M' stand for the molecular Marker, the 'S' stands for the wild-type recombinant HBeAg, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The concentrated protein was recovered from the filter and was stored in 1ml aliquots at -80°C . This purification process was followed for all the mutant proteins. Therefore, only the SDS PAGE gel pictures are presented below.

The -7S plasmid was electroporated in to top10 electro-competent cells. A three liter culture was grown and the protein was purified. The gels below shows the various stages of purification.

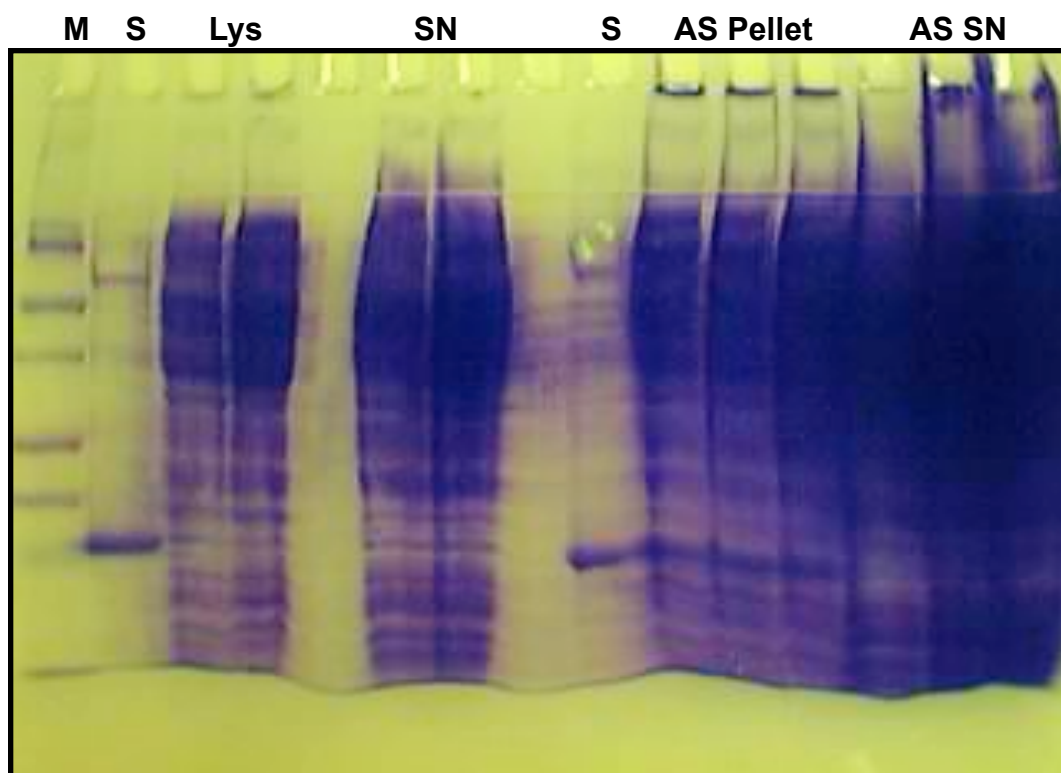


Figure 9. **SDS PAGE Gel picture of -7S.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'SN' stands for supernatant, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

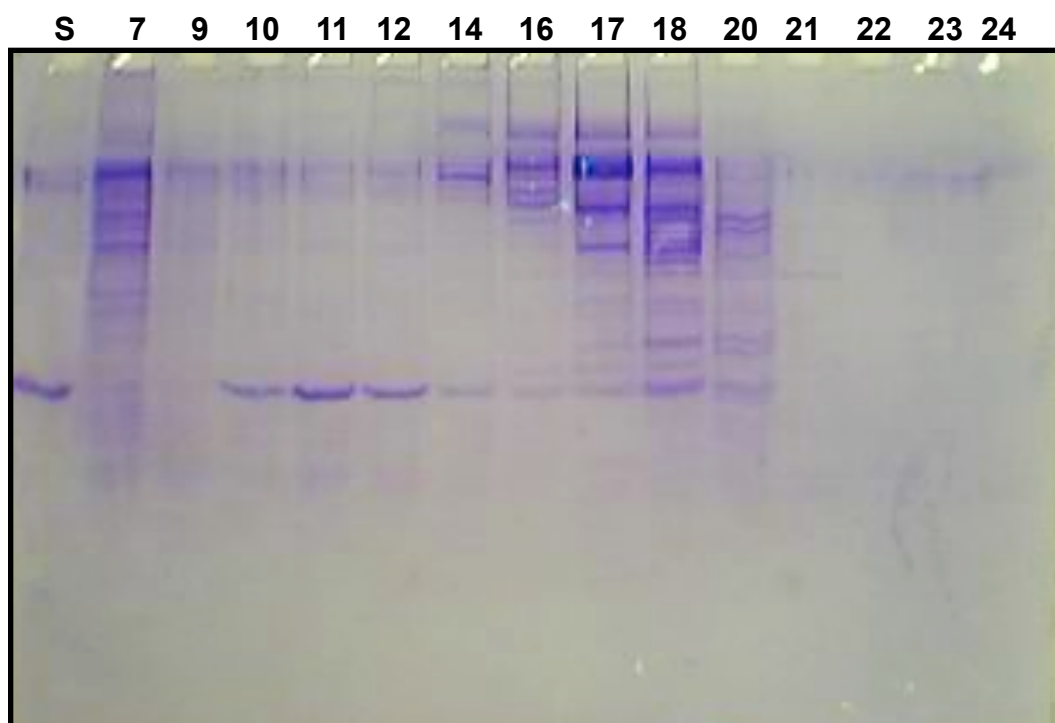


Figure 10. **SDS PAGE Gel picture of fractions of -7S.** The fractions collected of the sizing column were run on the gel.

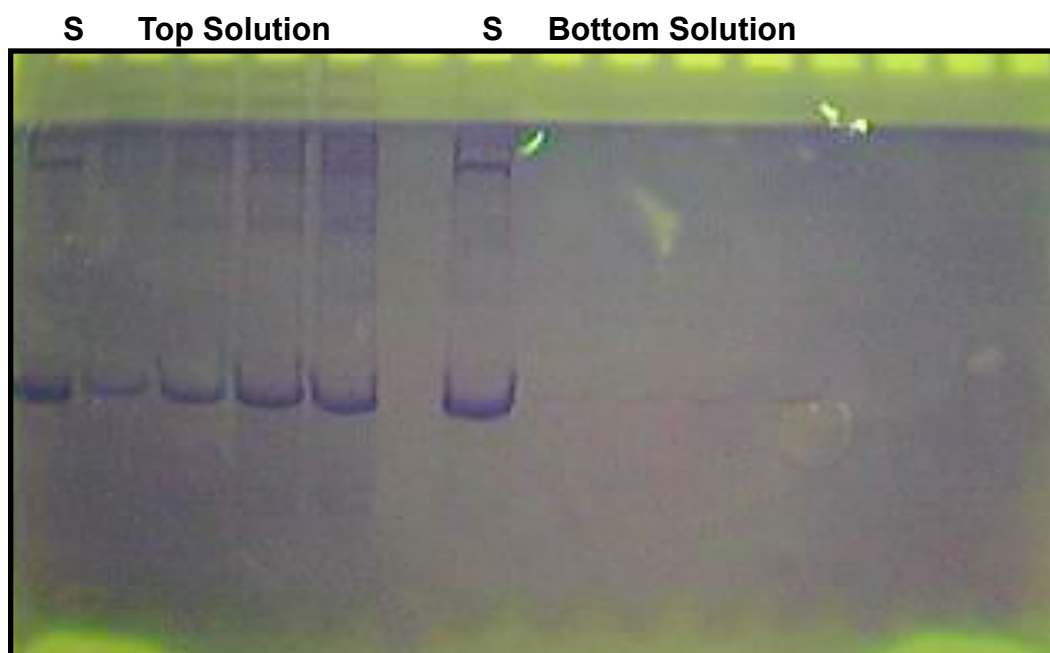


Figure 11. **SDS PAGE Gel picture of concentrated -7S.** The 'M' stand for the molecular Marker, the 'S' stands for wild-type recombinant HBeAg, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The -7S A plasmid was electroporated in to top10 electro-competent cells. A three liter culture was grown and the protein was purified. The gels below shows the various stages of purification.

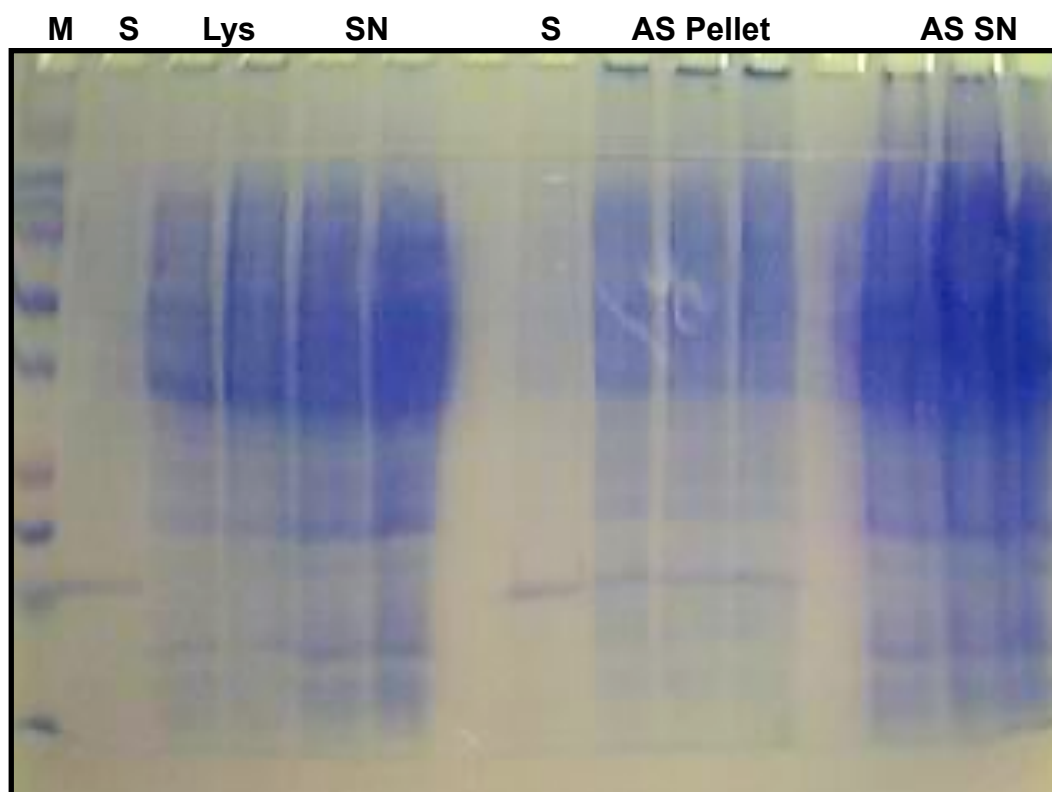


Figure 12. **SDS PAGE Gel picture of -7S A.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'SN' stands for supernatant, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

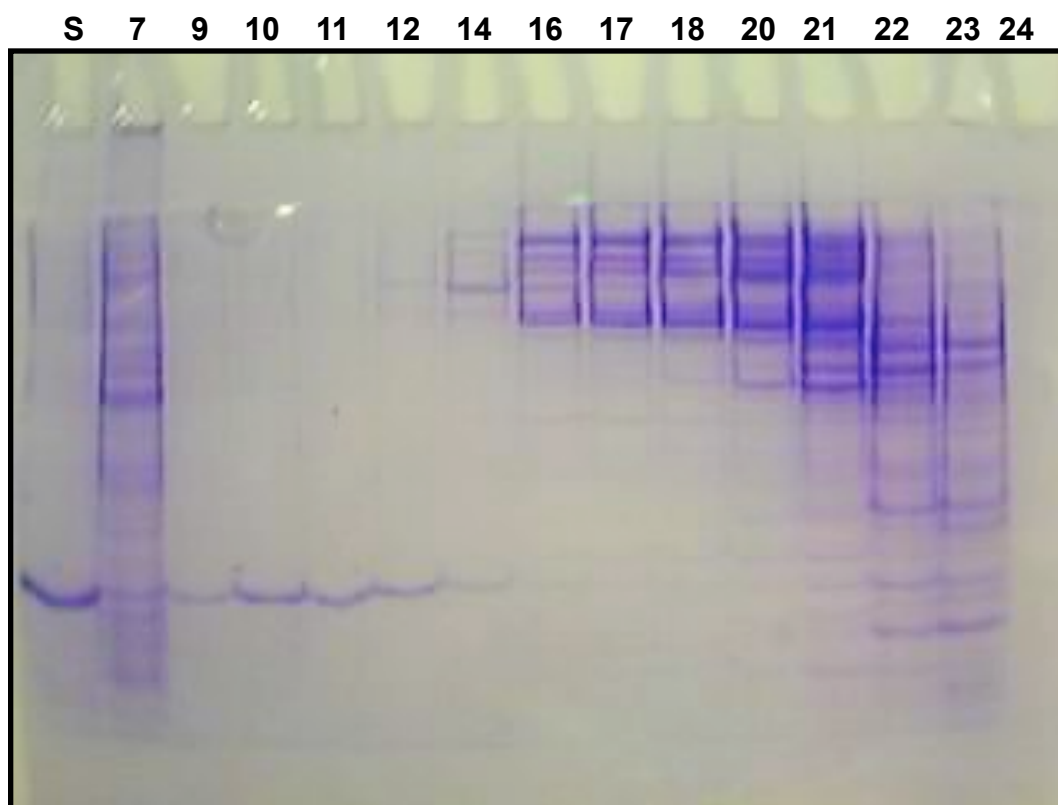


Figure 13. **SDS PAGE Gel picture of fractions of -7S A.** The fractions collected of the sizing column were run on the gel.

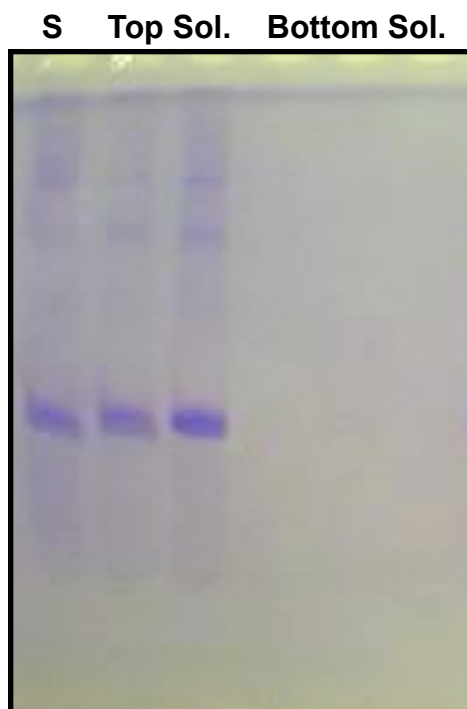


Figure 14. **SDS PAGE Gel picture of concentrated -7S A.** The 'S' stands for the wild type, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The -7S B plasmid was electroporated in to top10 electro-competent cells. A three liter culture was grown and the protein was purified. The gel below shows the various stages of purification.

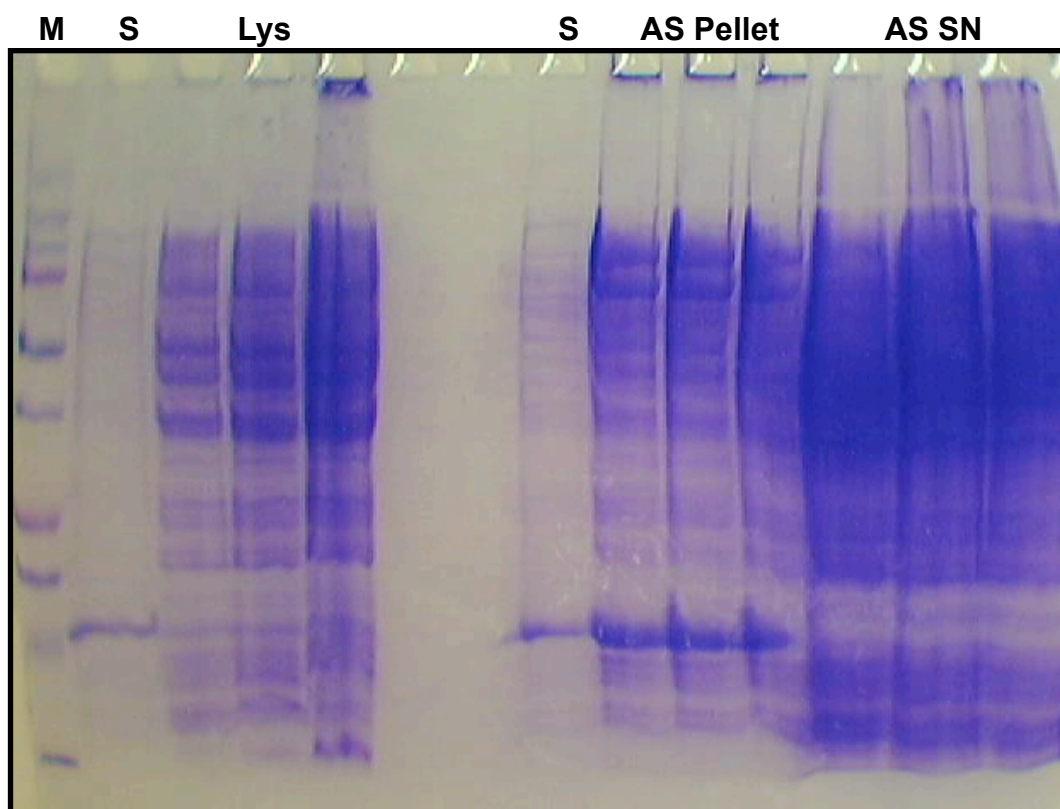


Figure 15. **SDS PAGE Gel picture of -7S B.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

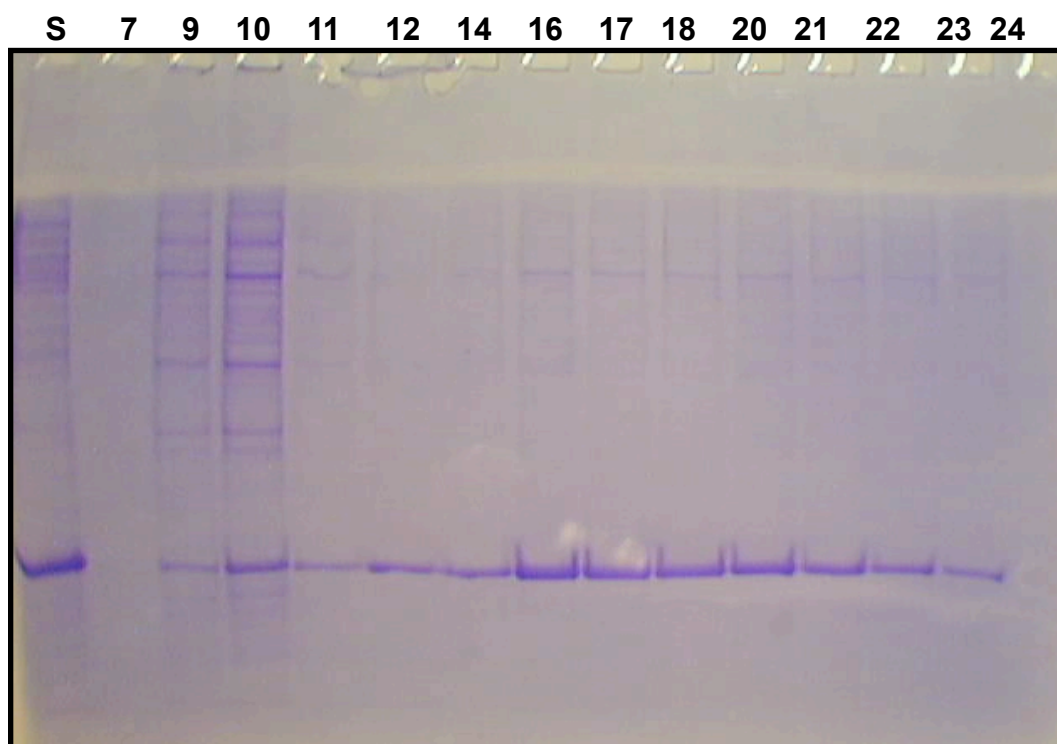


Figure 16. **SDS PAGE Gel picture of fractions of -7S B.** The fractions collected of the sizing column were run on the gel.

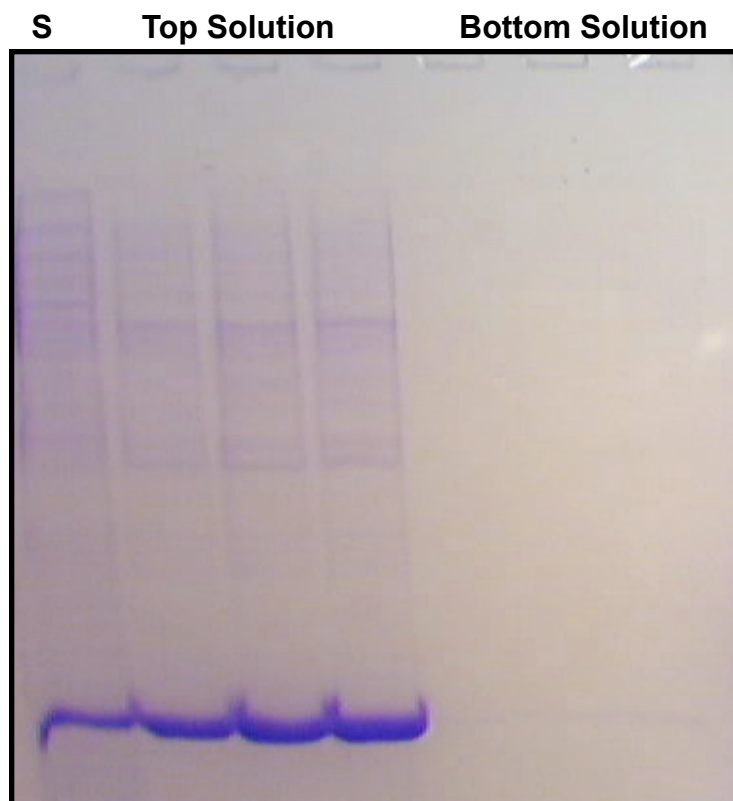


Figure 17. **SDS PAGE Gel picture of concentrated -7S B.** The 'S' stands for the wild type, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The -7S C plasmid was electroporated in to TB1 electro-competent cells. A three liter culture was grown and the protein was purified. The gel below shows the various stages of purification.

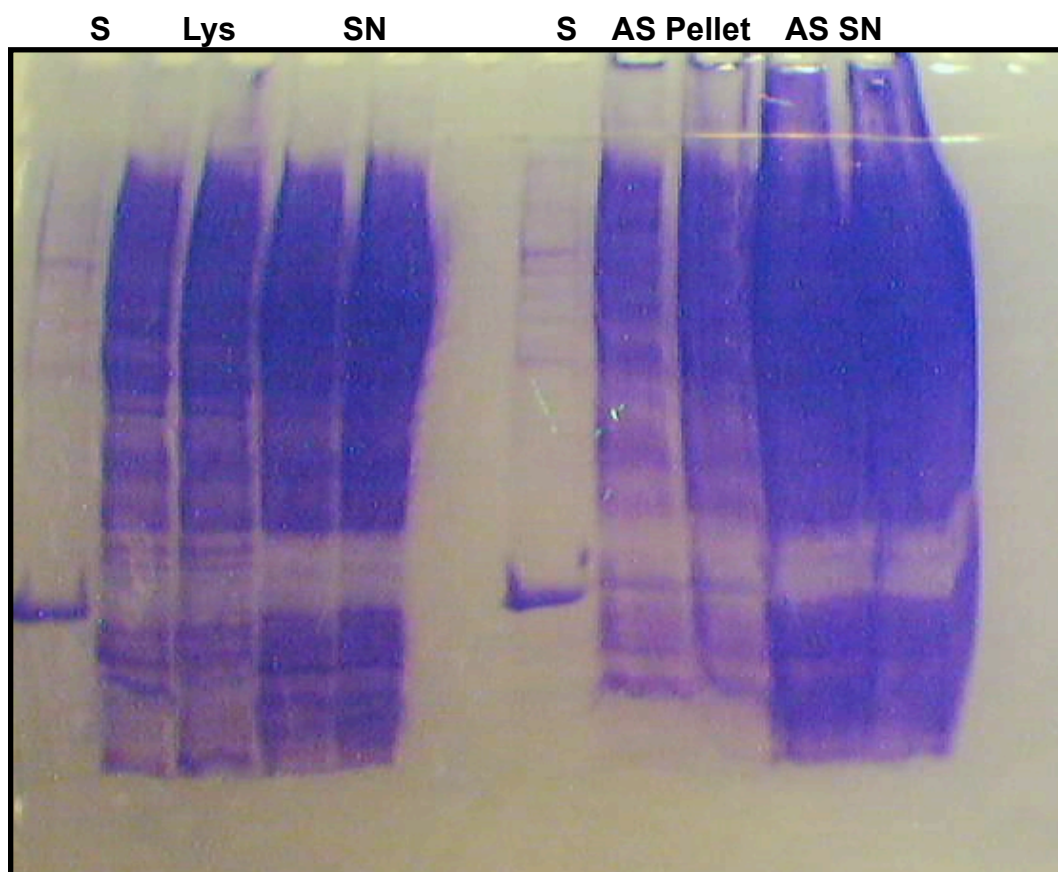


Figure 18. **SDS PAGE Gel picture of -7S C.** 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'SN' stands for supernatant, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

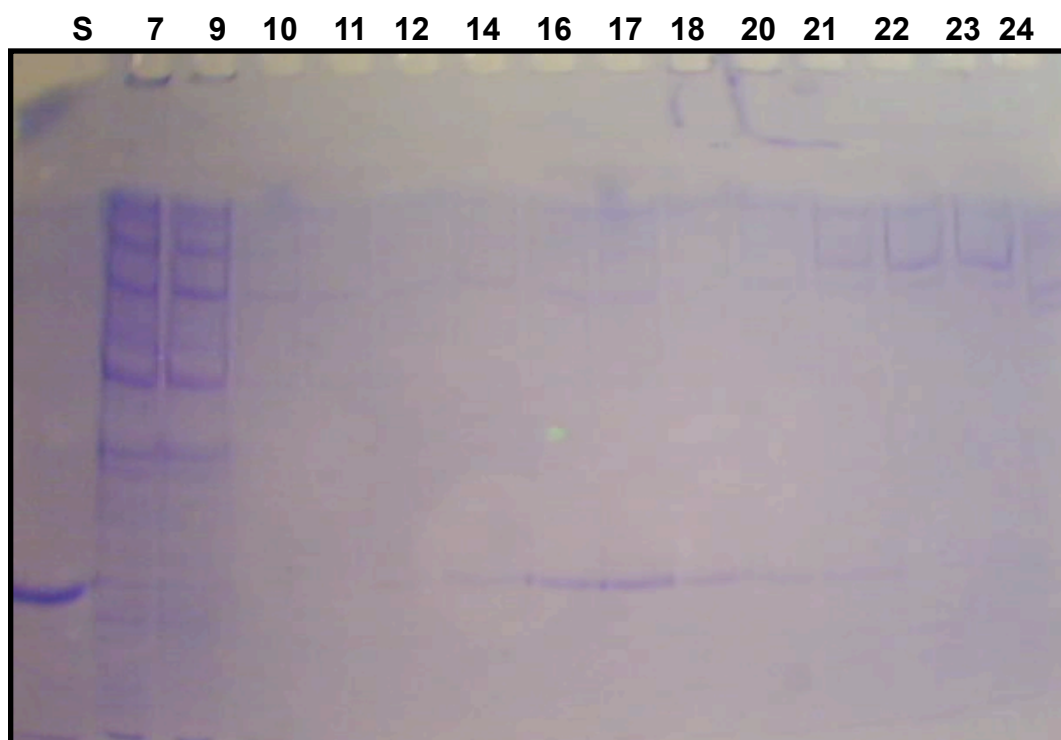


Figure 19. **SDS PAGE Gel picture of fractions of -7S C.** The fractions collected of the sizing column were run on the gel.

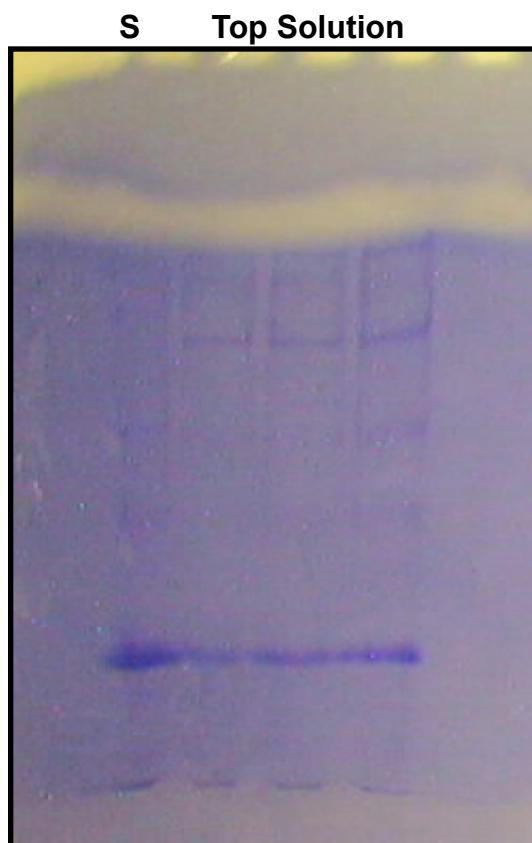


Figure 20. **SDS PAGE Gel picture of concentrated -7S C.** 'S' stands for wild-type recombinant HBeAg, the 'Top solution' is the solution which retained in the concentrator. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The PCA plasmid was electroporated in to TB1 electro-competent cells. A three liter culture was grown and the protein was purified. The gel below shows the various stages of purification.

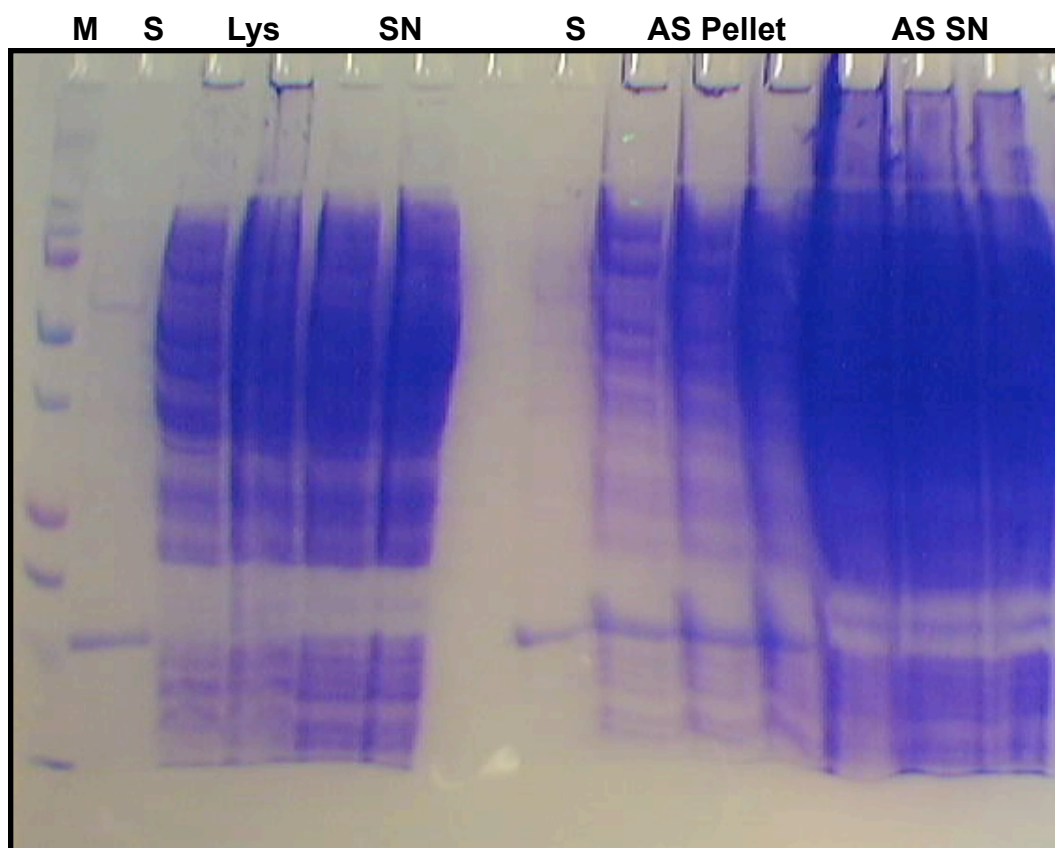


Figure 21. **SDS PAGE Gel picture of PCA.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'SN' stands for supernatant, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

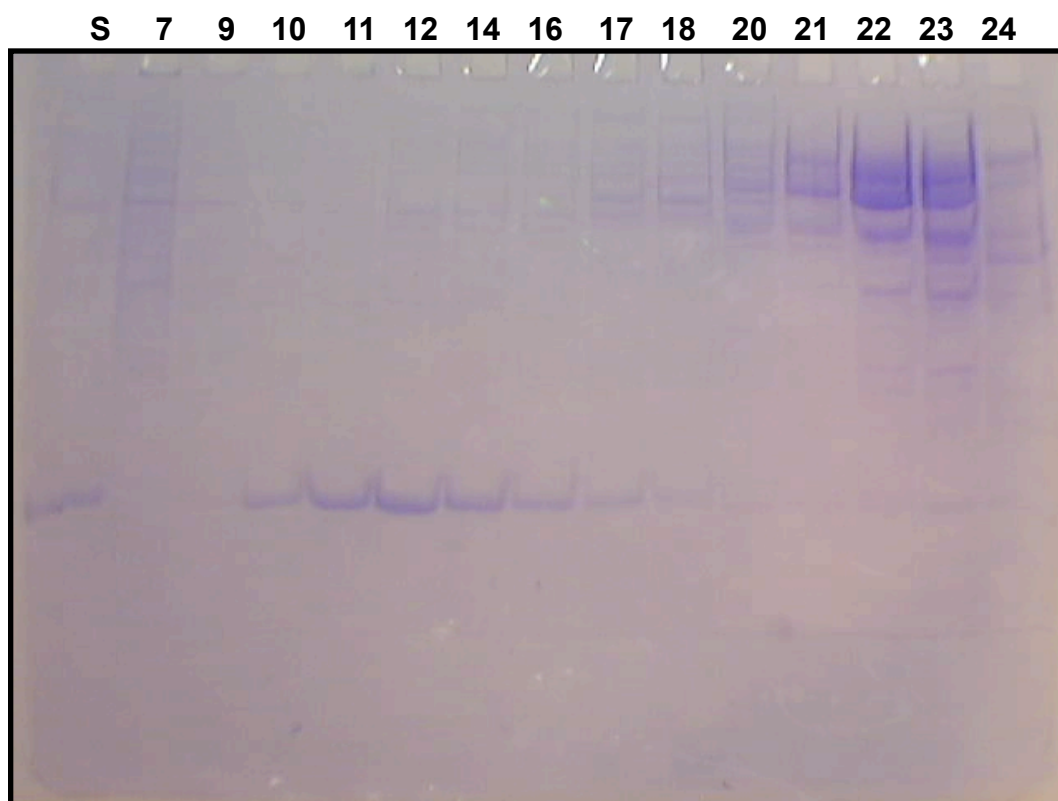


Figure 22. **SDS PAGE Gel picture of fractions of PCA.** The fractions collected of the sizing column were run on the gel.

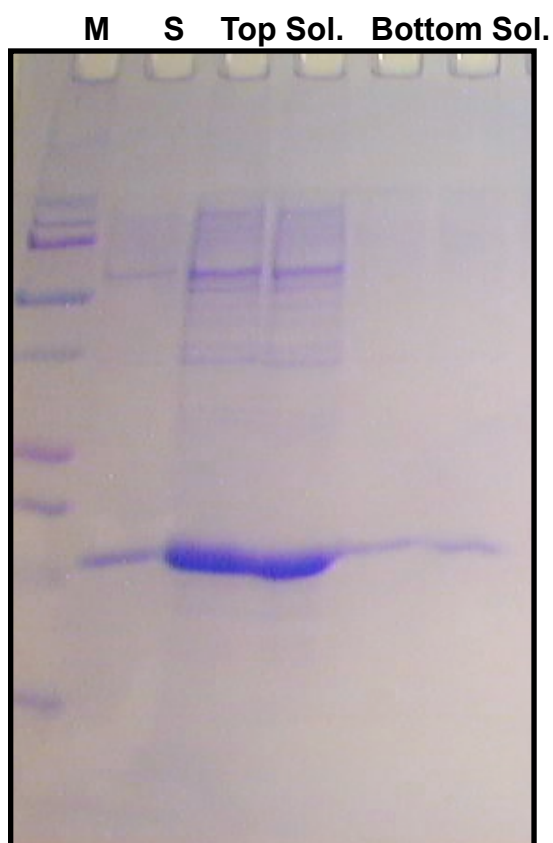


Figure 23. **SDS PAGE Gel picture of concentrated PCA.** The 'M' stand for the molecular Marker, the 'S' stands for wild-type recombinant HBeAg, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The PCB plasmid was electroporated in to TB1 electro-competent cells. A three liter culture was grown and the protein was purified. The gel below shows the various stages of purification.

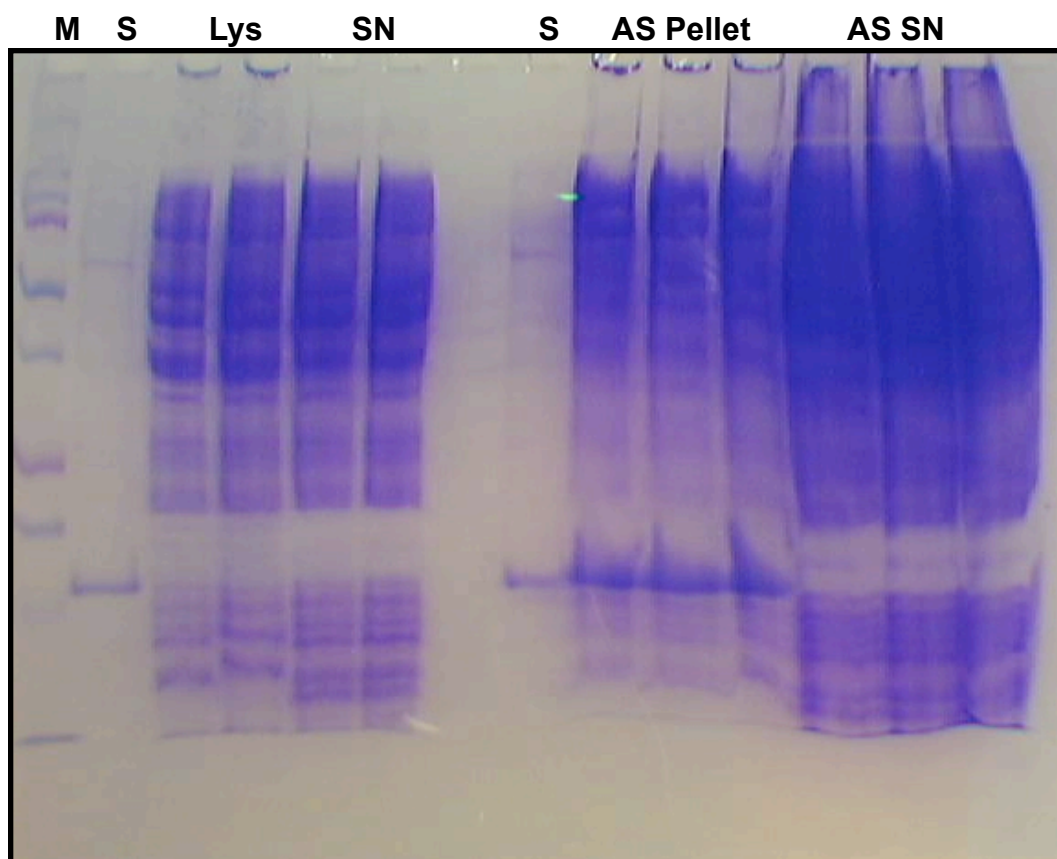


Figure 24. **SDS PAGE Gel picture of PCB.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'SN' stands for supernatant, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

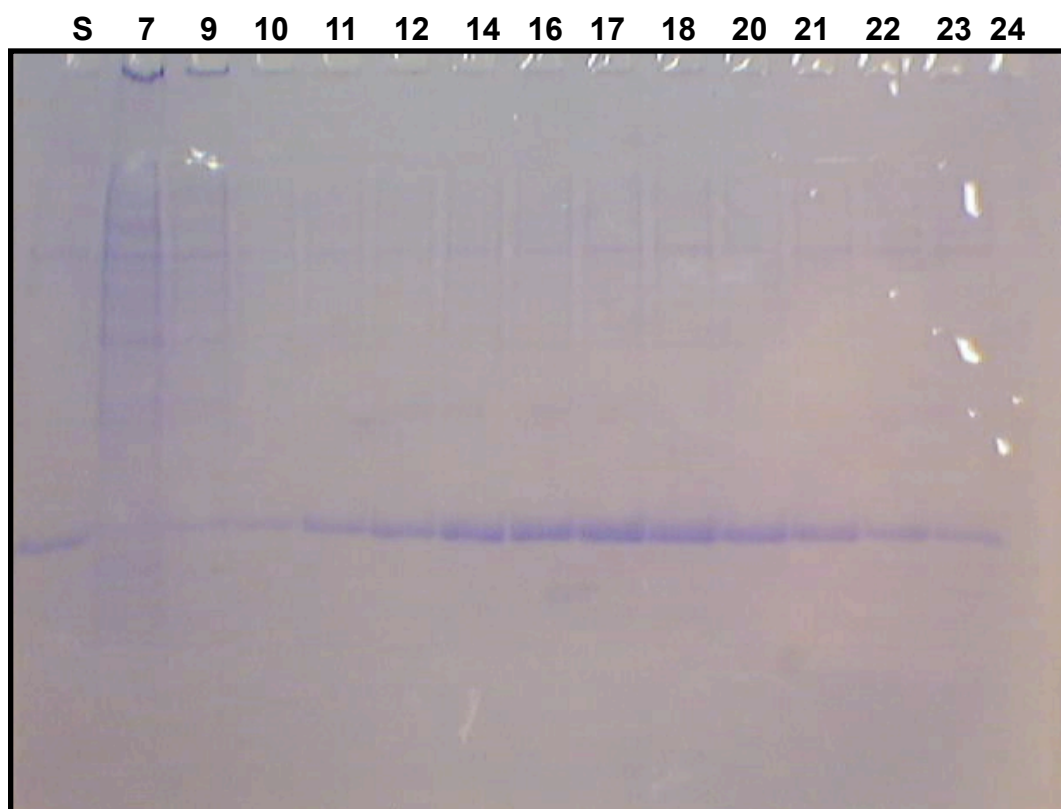


Figure 25. **SDS PAGE Gel picture of fractions of PCB.** The fractions collected of the sizing column were run on the gel.

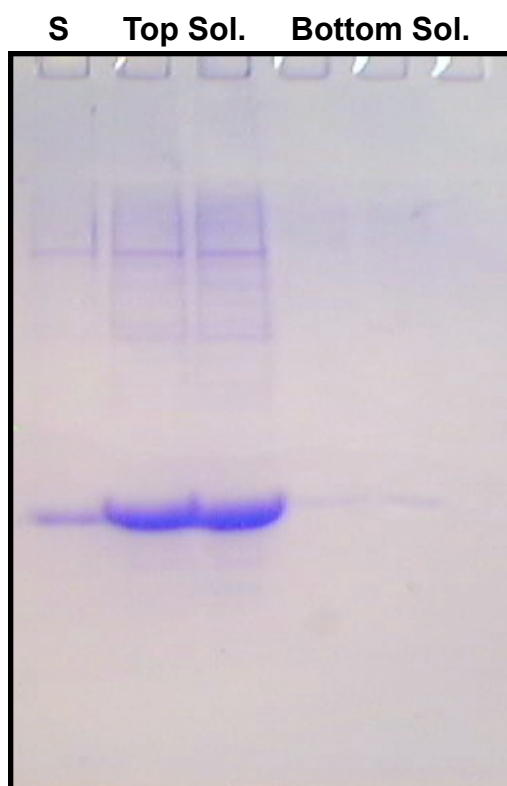


Figure 26. **SDS PAGE Gel picture of concentrated PCB.** 'S' stands for wild-type recombinant HBeAg, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

The PCC plasmid was electroporated in to TB1 electro-competent cells. A three liter culture was grown and the protein was purified. The gel below shows the various stages of purification.

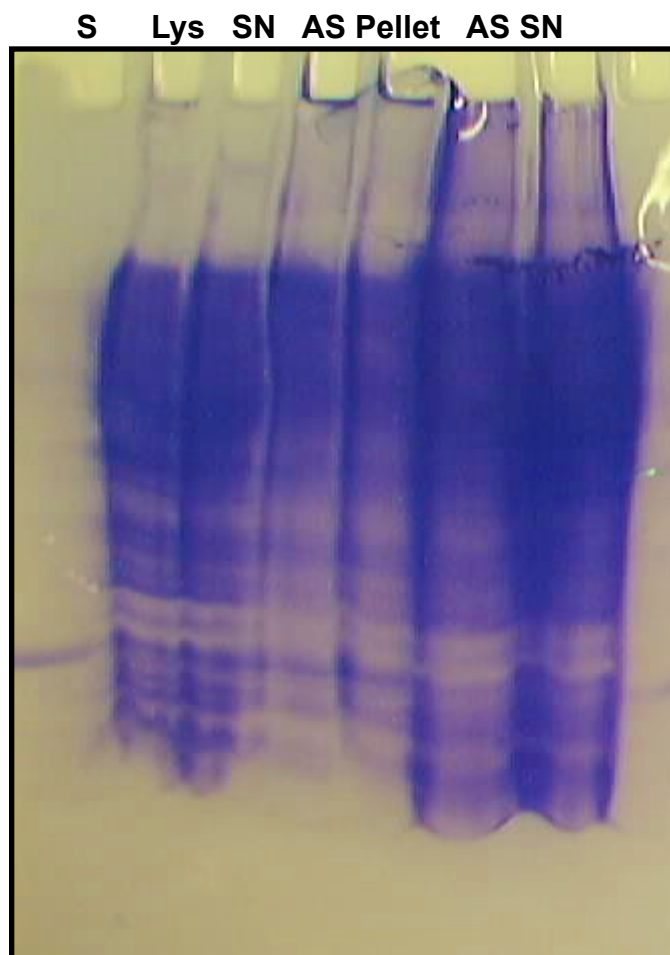


Figure 27. **SDS PAGE Gel picture of PCC.** 'M' stands for Molecular Marker, 'S' stands for wild-type recombinant HBeAg, 'Lys' stands for Lysate, 'SN' stands for supernatant, 'AS Pellet' stands for 20% ammonium sulfate pellet and 'AS SN' stands for 20% ammonium sulfate supernatant.

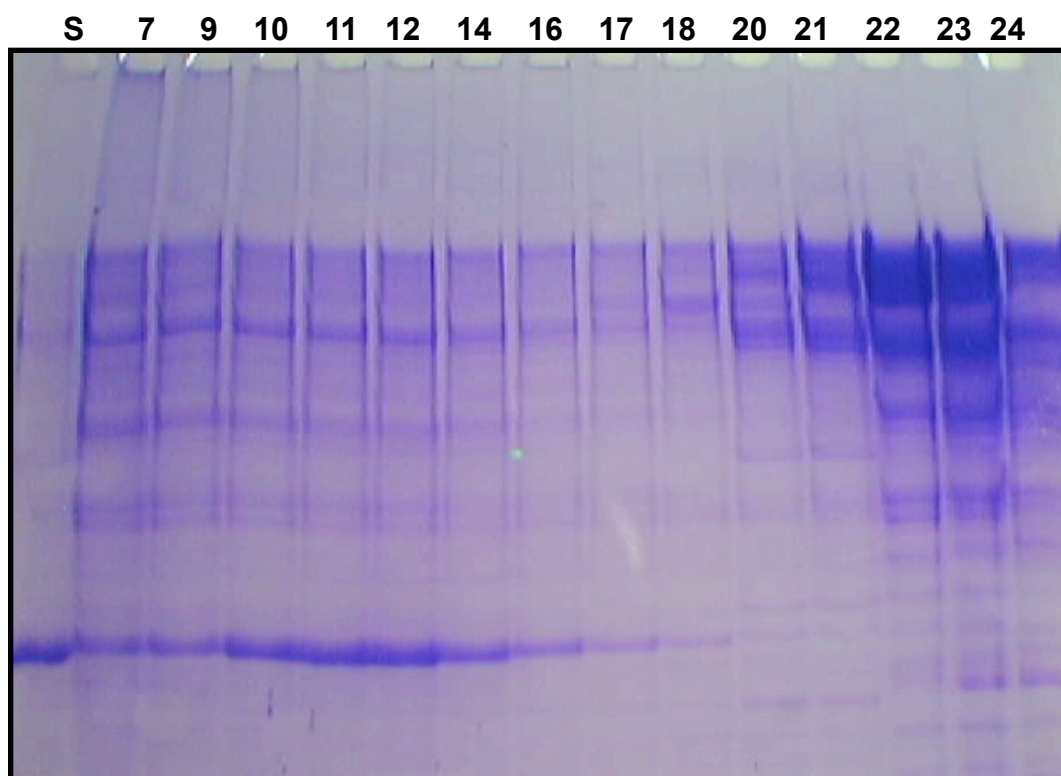


Figure 28. **SDS PAGE Gel picture of fractions of PCC.** The fractions collected of the sizing column were run on the gel.

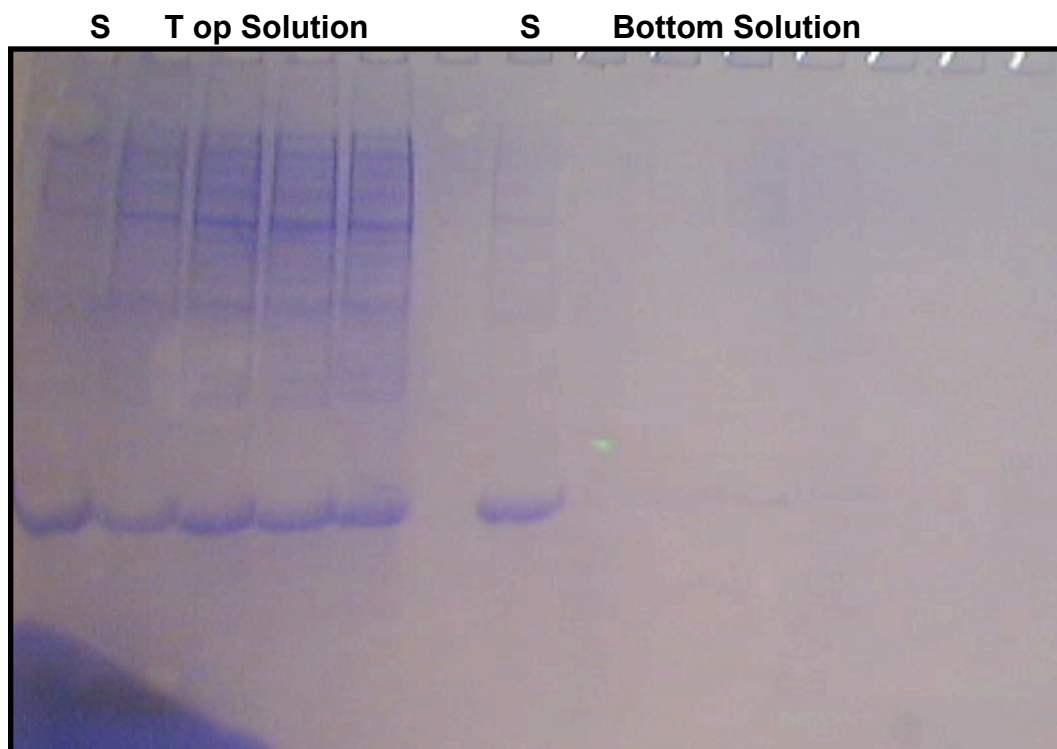


Figure 29. **SDS PAGE Gel picture of concentrated PCC.** 'S' stands for wild-type recombinant HBeAg, the 'Top solution' is the solution which retained on the concentrator and the 'Bottom solution' is the solution passed through the ultra filter. The top as well as bottom solutions were loaded in increasing concentrations in different wells.

An important aspect to be noted here is that the expression levels of the wild-type recombinant HBeAg as well as all the mutants differed up to a certain extent. The following is the table showing the differences in total concentrations obtained amongst the mutants from a 3 liter batch.

These wild-type recombinant HBeAg, -7S, -7S A, -7S B, -7S C, PCA, PCB and PCC were stored at concentrations of 1.6mg, 0.98mg, 0.85mg, 1.1mg, 0.9mg, 1mg and 1.3mg in 1ml buffer respectively at 20°C until use.

Protein	Total Amount
E Antigen	12.8 mg
-7S	9.1 mg
-7S A	7.8 mg
-7S B	5.95 mg
-7S C	7.7 mg
PCA	6.3 mg
PCB	7 mg
PCC	9.1 mg

Table 2. **Table showing the total amounts of all the proteins obtained.**

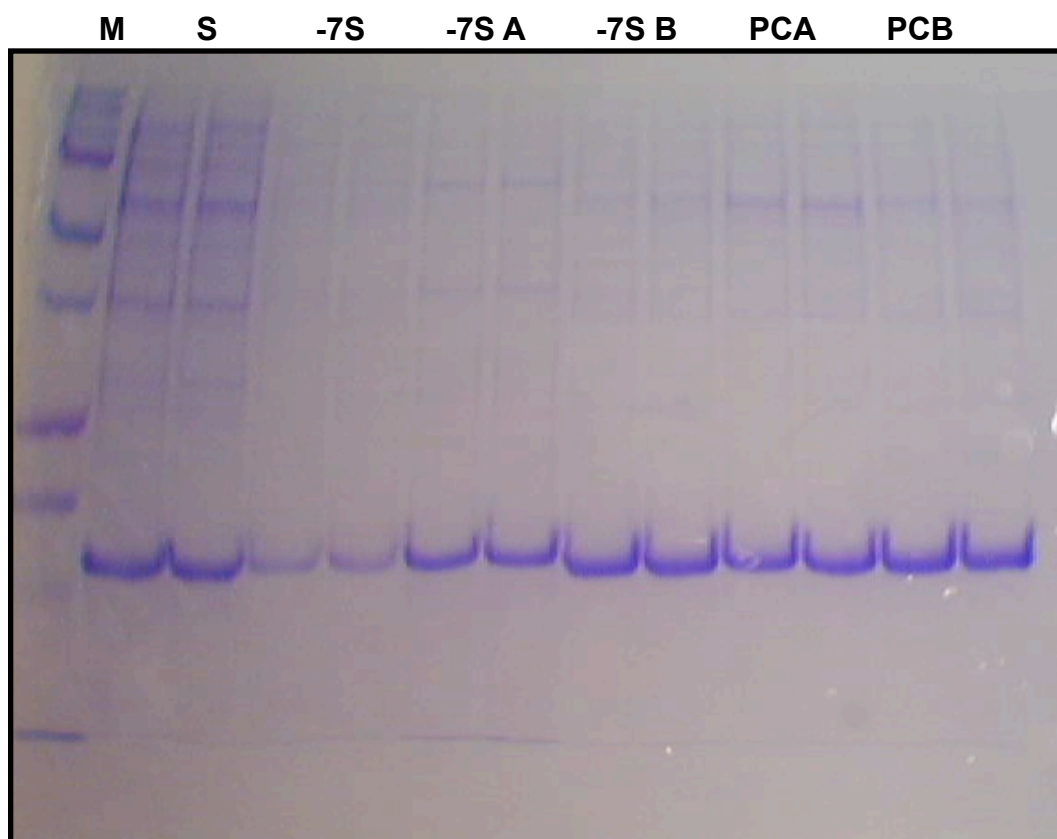


Figure 30. **SDS PAGE Gel showing same concentration of proteins loaded.** The first lane is the molecular marker followed by 2 lanes each of the wild-type recombinant HBeAg, -7S, -7S A, -7S B, PCA and PCB respectively.

From the above gel, it is clear that some mutants are more pure than others and they also differ in total concentration. Here equal concentration of all the mutants along with the wild-type HBeAg were loaded on an SDS PAGE gel. Two wells for each of the mutants and wild-type were loaded. From the gel it can be observed that the purity of the -7S mutant is the highest. It was almost 100% pure, but it had the lowest concentration. On the other hand, the wild-type HBeAg had the highest amounts of impurities and it was about 85% pure. Further, mutants -7S A and -7S B were around 95% pure whereas, mutants PCA and PCB were about 90% pure.

Evaluation of the Disulfide Bonding in HBeAg by 2D SDS PAGE

2D gel electrophoresis was used to determine the size distribution of the disulfide bonded components of wild type recombinant HBeAg. Two 12% gels were made. One gel was run with the wild-type recombinant HBeAg in the reduced form as well as multiple lanes of the non-reduced form. After the gel was run, one unstained lane of unreduced protein was cut from the gel. The remainder of the gel was stained to verify that the resolution of proteins was good. This strip containing the unreduced protein was placed horizontally on the top of another 12% gel. A 2% agarose solution was prepared containing β -mercaptoethanol and was added to the horizontally placed gel strip, completely immersing it. This was allowed to stand for 30 minutes in order to reduce the proteins in the strip. In addition to this strip, two lanes were provided in the second gel. One was used for molecular weight markers, and the second was used for fully reduced HBeAg. Electrophoresis was then performed as usual. This gel was then silver stained so even the small amounts of proteins on the gel could be seen. The following is the diagrammatic representation.

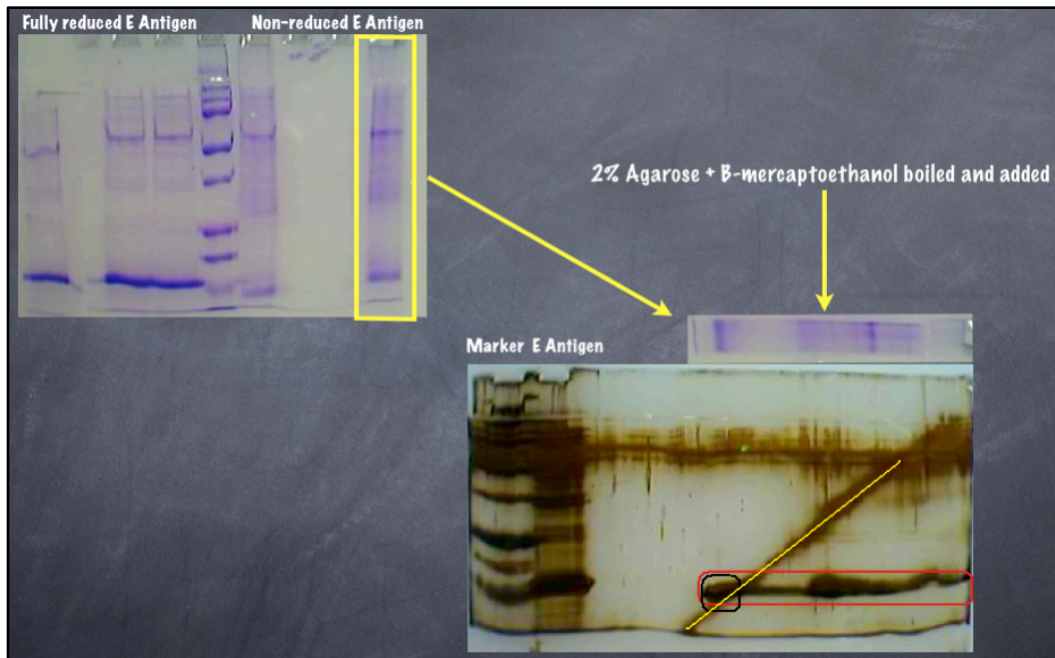


Figure 31. **2-D gel (non-reduced followed by reducing conditions) of recombinant HBeAg.** The proteins enclosed in the red box are the wild-type recombinant HBeAg protein. The protein in the black box is the wild-type recombinant HBeAg which migrates faster than monomers under non-reducing condition. The yellow diagonal shows proteins whose migration is unaffected by reduction.

The gel shown above is quite informative concerning the nature of the disulfide bonding within the wild-type recombinant HBeAg. Proteins that migrate with the same apparent molecular weight under reducing and non reducing conditions should be present along the diagonal line indicated in the figure (yellow line). However, it is clear that the HBeAg does not fall on this diagonal, but rather occurs at positions shown within the red box. This is the expected result if most of the protein was present in disulfide bonded oligomers greater than dimeric forms. In fact, as can be seen from the silver stained gel, the non-reduced strip which was cut out had the wild-type recombinant HBeAg throughout the whole strip. Based on this we would conclude that the wild-type recombinant HBeAg is present in forms ranging from monomers to

oligomers of increasing size, held together by disulfide bonds. In addition, one species appears to be present that runs faster than monomers, (denoted by the black box in the above figure) as evidenced by its position to the left of the diagonal. This would be expected to be a monomer with an intra-molecular disulfide bond, creating a less completely unfolded, and therefore more compact form of the protein. Upon reduction, this protein is then completely unfolded, and then migrates at the same position as expected for monomers (within the red box in the figure). Thus based on this figure, we would conclude that in addition to inter-molecular disulfide bonds, some of the protein has at least one intra-molecular disulfide bond.

ELISA

The antigenic activities of the wild type as well as all the mutants were checked. An ELISA plate was taken wherein the 1st and the 2nd wells were the controls without any protein added to it. The 6th well had the highest amount of protein and it was serially diluted up to the 3rd well. Assays were done in triplicates for each of the proteins and the results plotted along with standard error indicated.

The concentration of the wild-type recombinant HBeAg stock solution used for the ELISA was 1.6mg/ml. Out of the stock solution 14 μ g/10 μ l was taken for the assay.

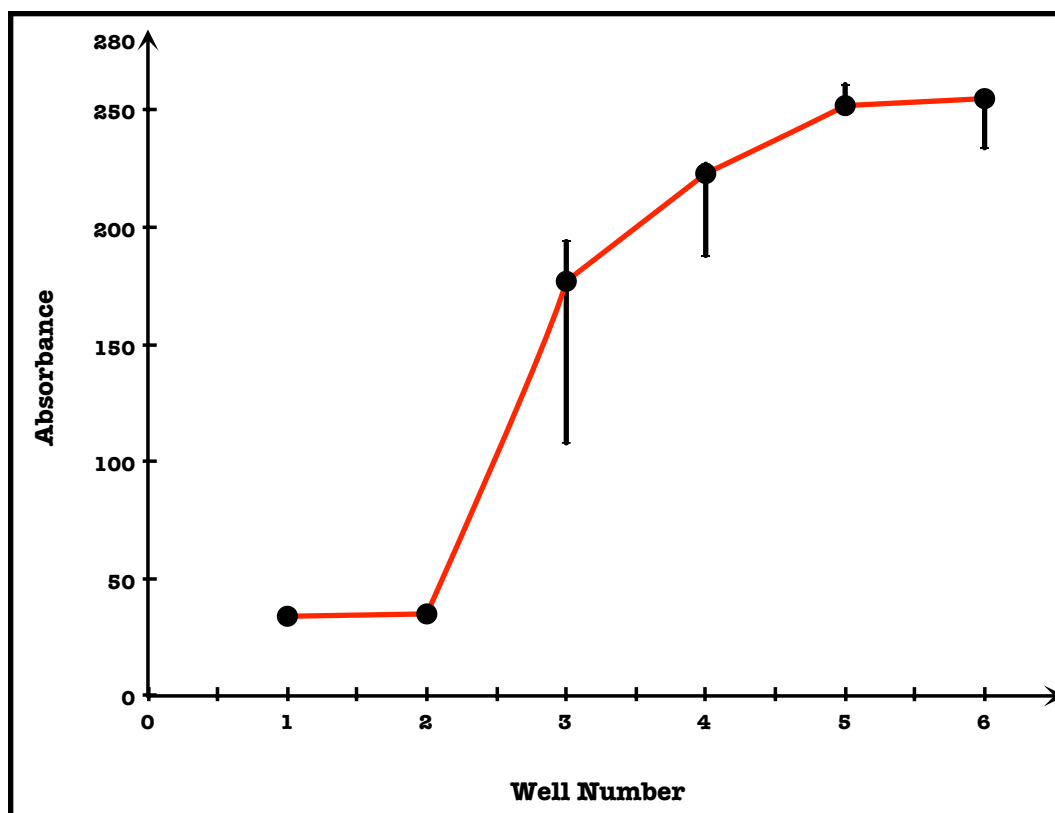


Figure 32. **Graph showing antigenic activity for wild-type recombinant HBeAg.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of -7S stock solution used for the ELISA was 1.3mg/ml. Out of the stock solution 17 μ g/10 μ l was taken for the assay.

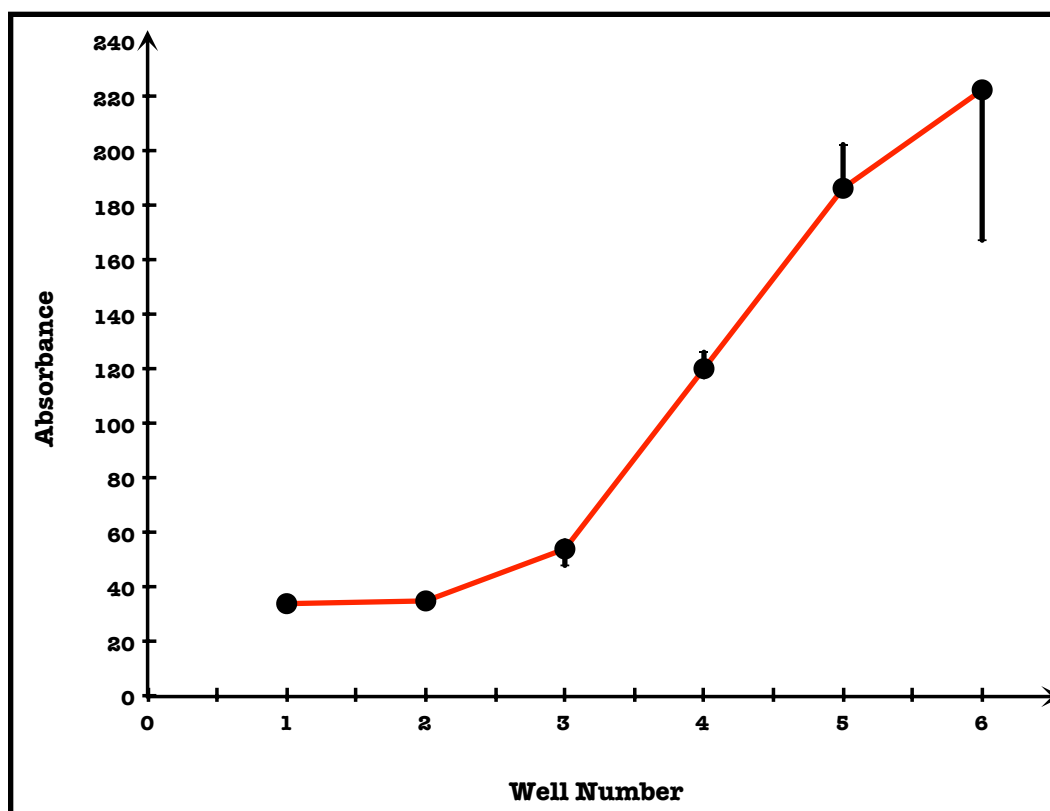


Figure 33. **Graph showing antigenic activity for -7S.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of -7S A stock solution used for the ELISA was 0.98mg/ml. Out of the stock solution 9.8 μ g/10 μ l was taken for the assay.

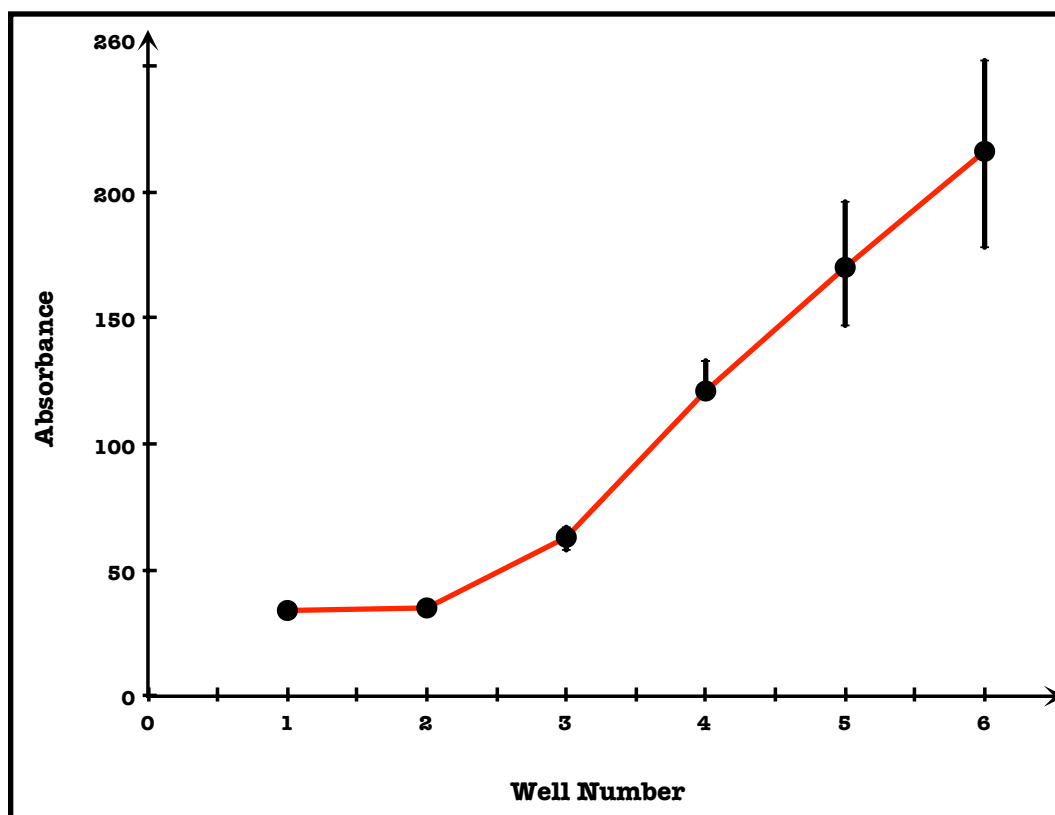


Figure 34. **Graph showing antigenic activity for -7S A.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of -7S B stock solution used for the ELISA was 0.85mg/ml.

Out of the stock solution 8.5µg/10µl was taken for the assay.

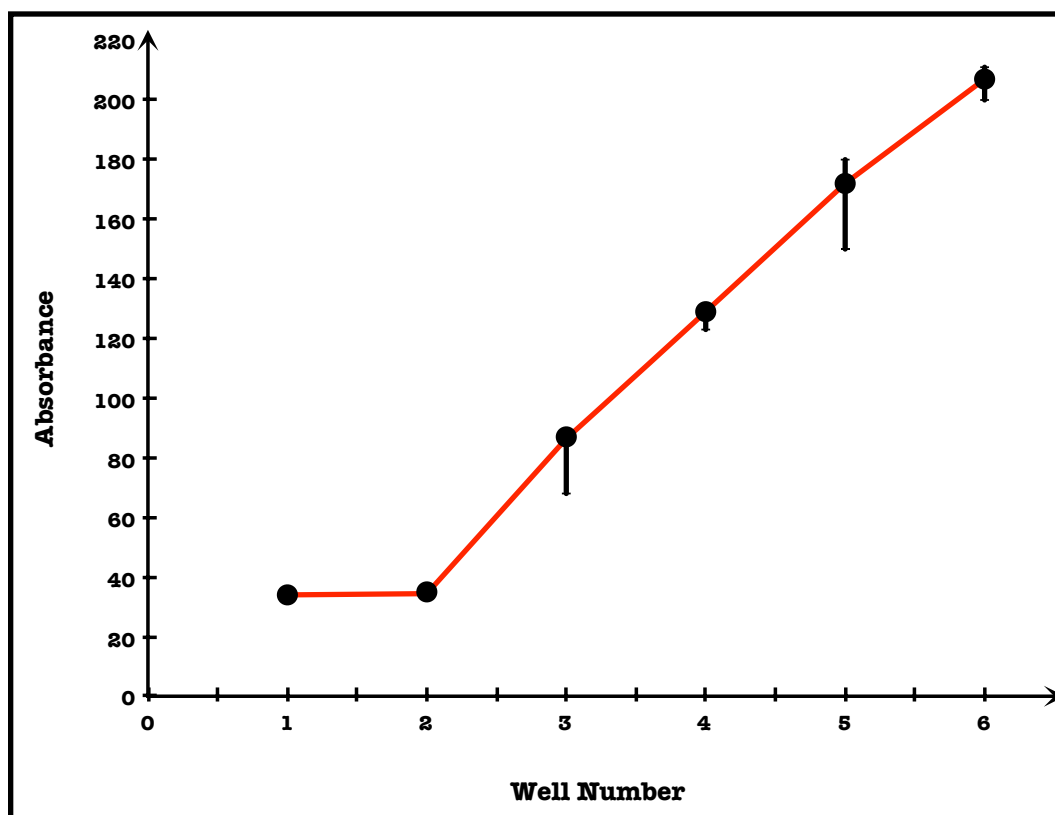


Figure 35. **Graph showing antigenic activity for -7S B.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of -7S C stock solution used for the ELISA was 1.1mg/ml. Out of the stock solution 11 μ g/10 μ l was taken for the assay.

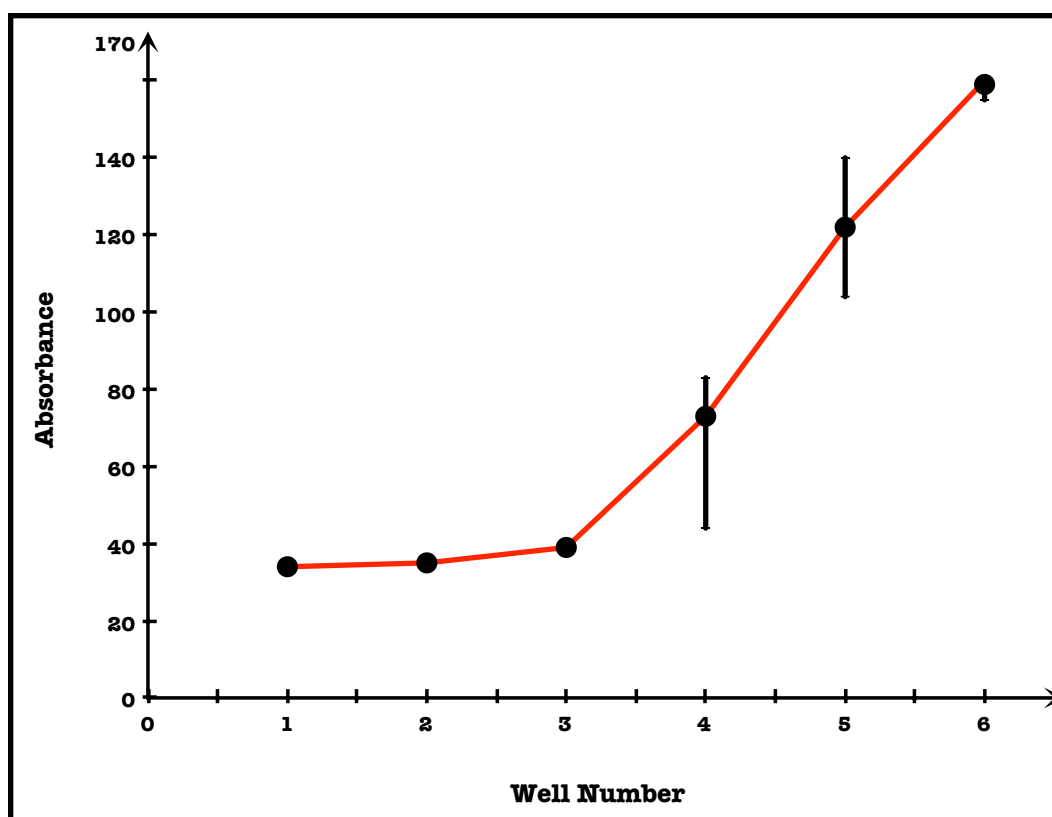


Figure 36. **Graph showing antigenic activity for -7S C.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of PCA stock solution used for the ELISA was 0.9mg/ml. Out of the stock solution 9 μ g/10 μ l was taken for the assay.

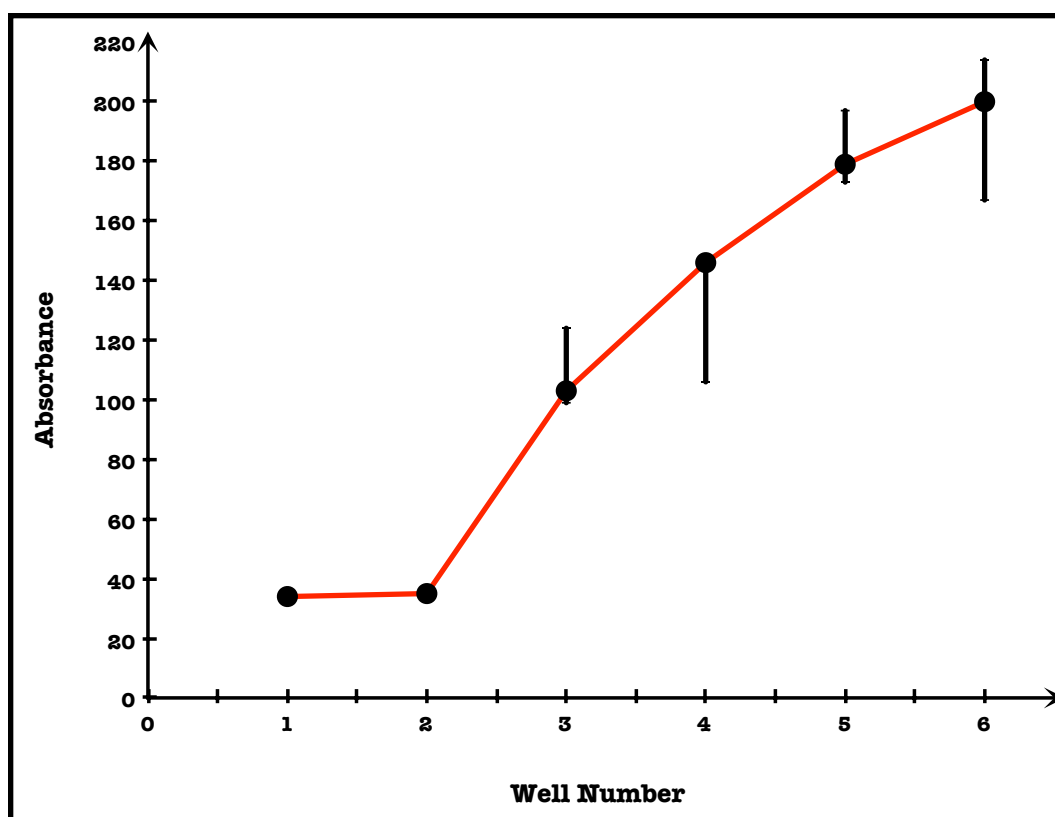


Figure 37. **Graph showing antigenic activity for PCA.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of PCB stock solution used for the ELISA was 1mg/ml. Out of the stock solution 10 μ g/10 μ l was taken for the assay.

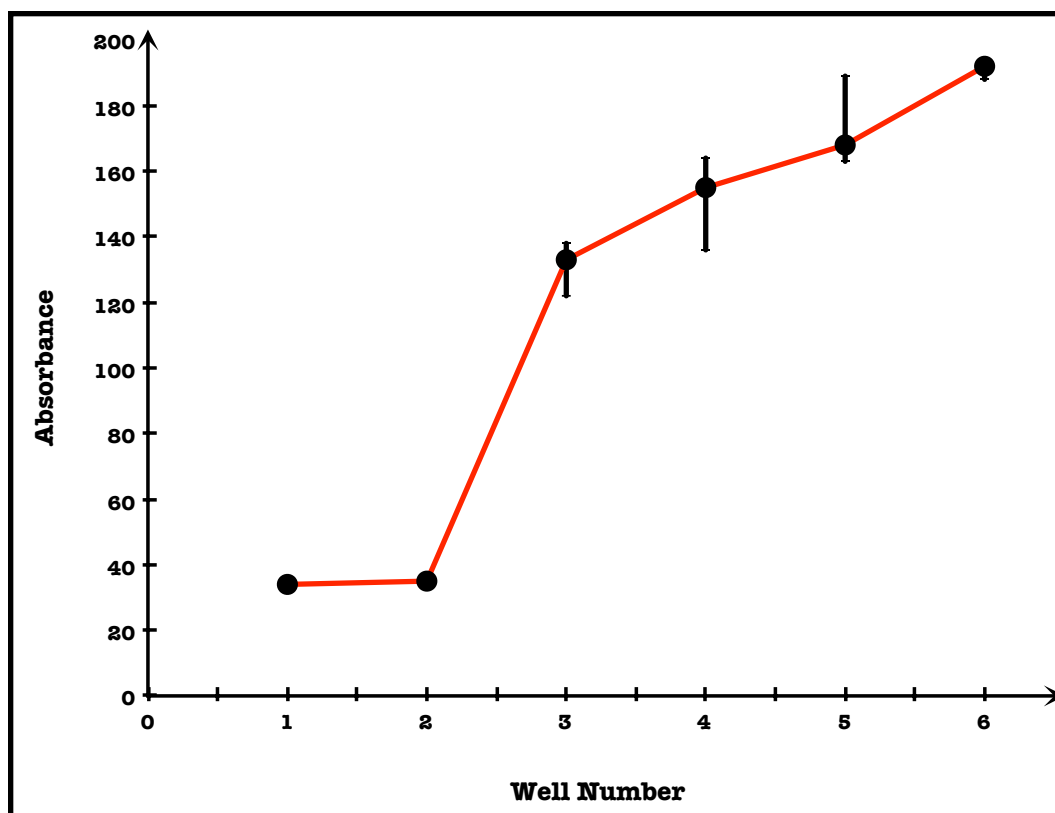


Figure 38. **Graph showing antigenic activity for PCB.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

The concentration of PCC stock solution used for the ELISA was 1.3mg/ml. Out of the stock solution 13 μ g/10 μ l was taken for the assay.

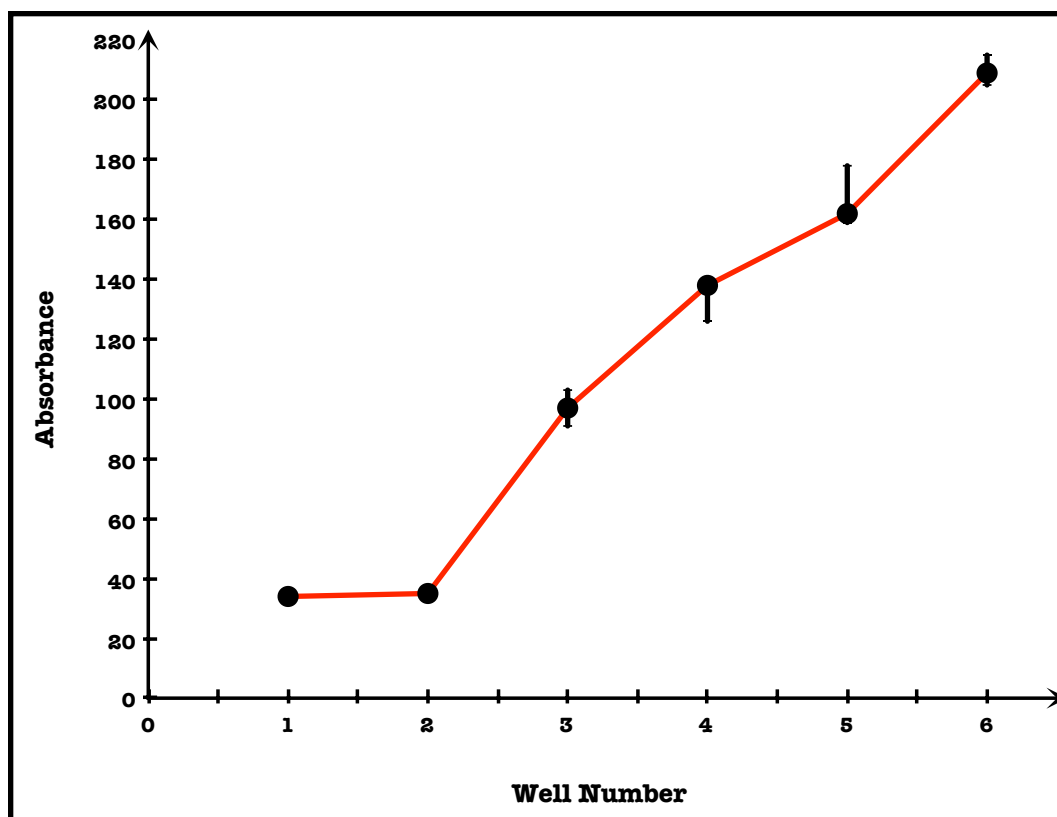


Figure 39. **Graph showing antigenic activity for PCC.** Well number 1 and 2 are the controls and contain no protein. Well number 6 contains the highest amount of protein and it is further serially diluted up to well 3. The dilution factor from well 6 to well 3 is 1:10, 1:100, 1:1000, 1:10000 respectively. The dotted lines are the error bars.

From all of the above assays we would conclude that all of the proteins exhibited high reactivity with the polyclonal antibodies used in these assays. There are variations observed amongst the proteins, however much of these variations are due to the differences in the amounts of protein used for the assay. The variations also could be due to some differences in the purity of the proteins. Whether there are real differences in the antigenic activity is difficult to determine using this polyclonal antibody. It would

be necessary to perform more assays with lower dilution factors to really quantitate the immunogenicity. Moreover, the use of more defined monoclonal antibodies would permit more definitive conclusions concerning the nature of the antigenicity. However, for the current studies, it is sufficient to be able to conclude that the isolated proteins do have high antigenic activity.

Reduction or Reduction and Alkylation of HBeAg

Two gels were run for the wild-type recombinant HBeAg and other mutants, one gel was run with samples that had only been reduced, and the other was run with samples that had been reduced and alkylated using iodoacetamide. In all of the following gels, lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the last lane contains no reducing agent.

Wild-type recombinant HBeAg:

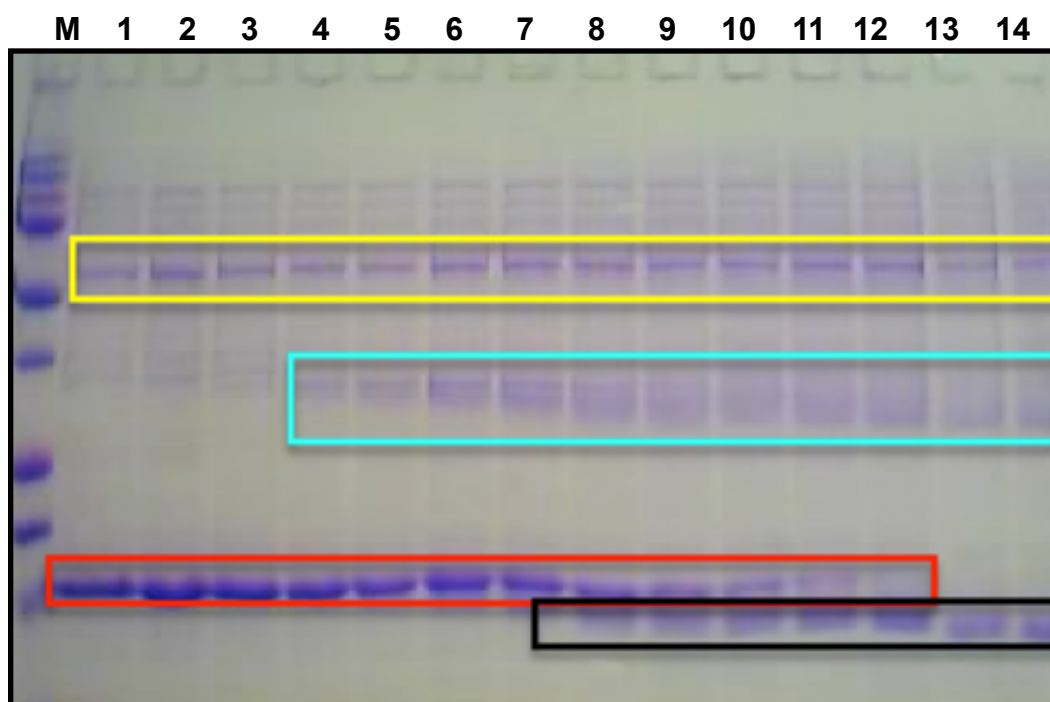


Figure 40. **Effect of decreasing the concentration of reducing agent on wild-type recombinant HBeAg without iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

In the gel shown above, one can see that under fully reduced conditions, the protein runs as the expected monomer (indicated by the red box). Under decreasing levels of reducing agent, the amount of monomer decreases. This is accompanied by an increase in the dimeric species observed (as indicated in the blue box above) and the appearance of a band that moves faster than monomers. (as shown in the black box above). This is consistent with the observations on the previous 2D gel. In addition there is a minor contaminant protein shown in the yellow box.

On the gel shown below, where the proteins were reduced and alkylated prior to being run on the SDS gel, the results are somewhat different. It is now clear that the majority of the protein fails to enter the gel, (as indicated in the black box) but rather remains in the sample well, because it is too large. We would conclude from this that the wild-type recombinant HBeAg is present mostly as a highly disulfide cross-linked macromolecular structure.

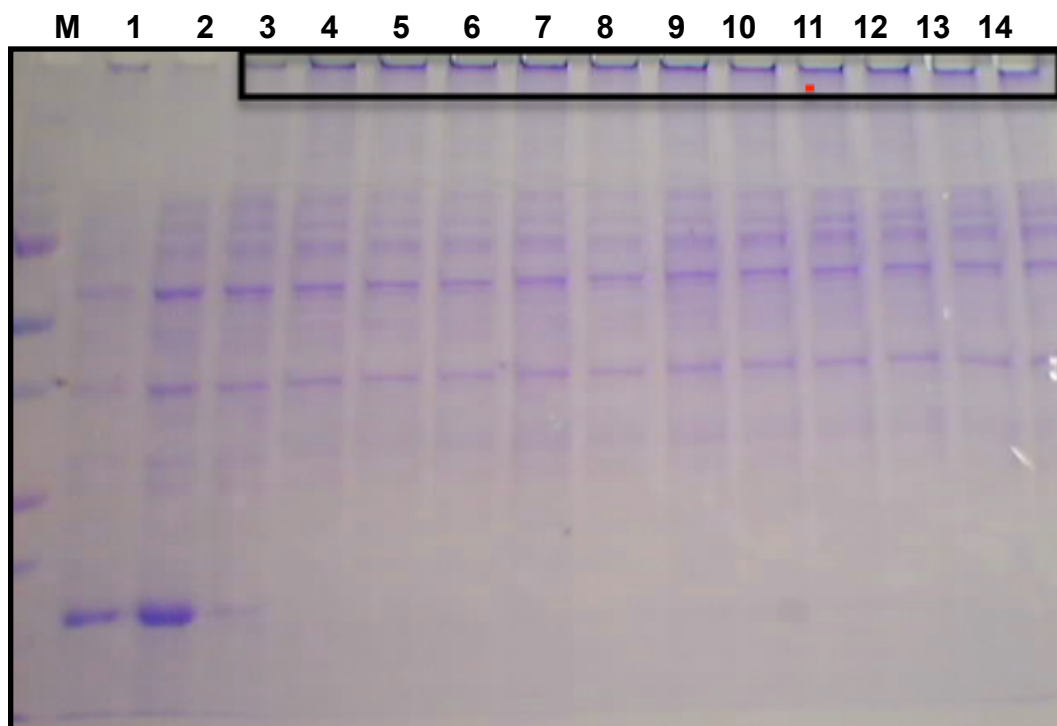


Figure 41. **Effect of decreasing the concentration of reducing agent on wild-type recombinant HBeAg with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

The model shown below would account for these observations. Disulfide bonding between cysteines within the core antigen sequence (residues 48 and 61) are known to disulfide bond as shown. If the -7C of the pre-core were to form additional disulfide bonds as shown, the result would be a high molecular weight oligomeric structure.

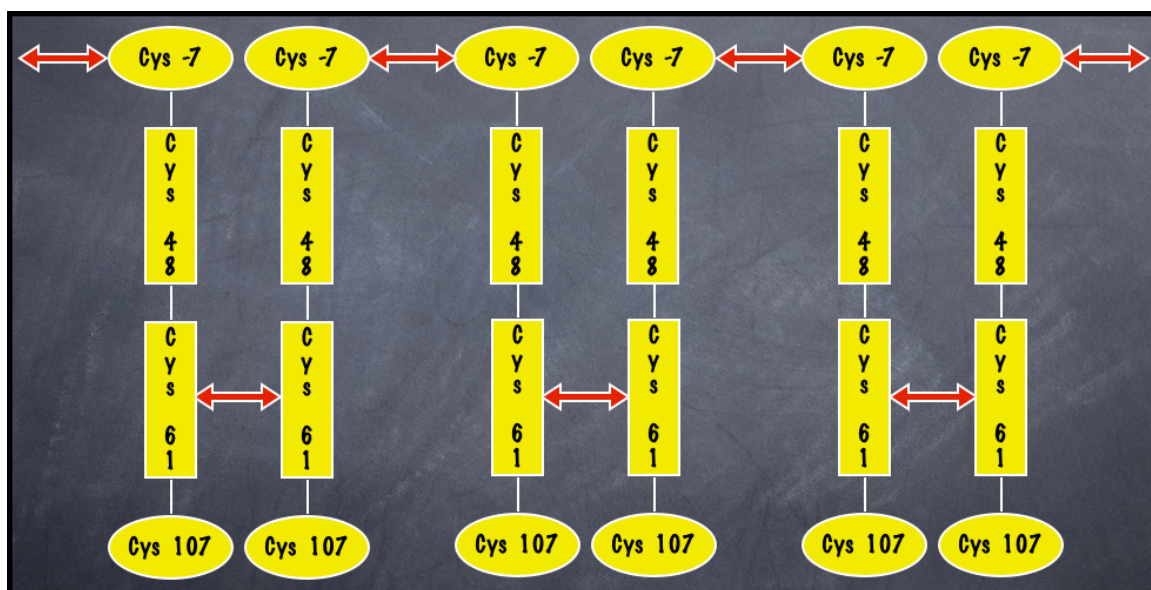


Figure 42. **Inter-molecular disulfide bond formation observed from figure 41.**

As can be seen from figure 41 that as the concentration of reducing agent decreases from left to right, the band at the bottom of the gel disappears showing that there are no monomers. Moreover, with decreasing concentration of reducing agent, the wild-type recombinant HBeAg mostly stays on top on the wells since probably it is too large to enter the gel. Therefore, it is possible that a chain is formed connecting a lot of particles together.

The gel below shows the results of similar studies with the -7S protein. In both cases, reduced or reduced and alkylated, the results do not show the presence of such highly cross-linked protein. This is most readily seen in the reduced and alkylated samples.

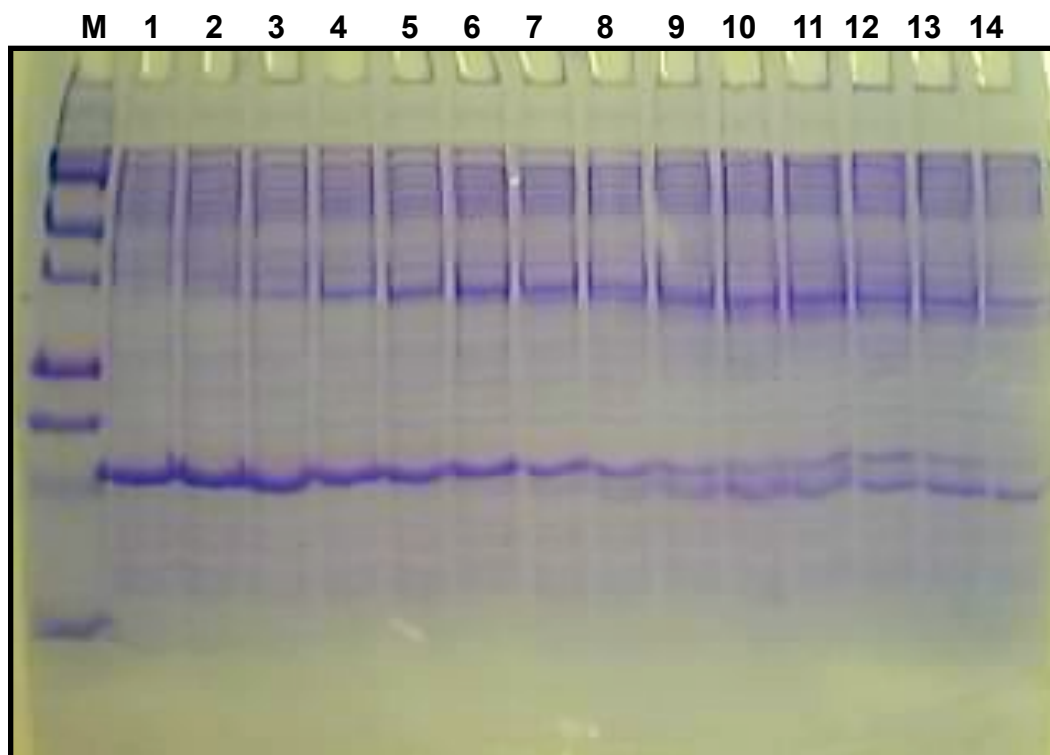


Figure 43. **Effect of decreasing the concentration of reducing agent on -7S without iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

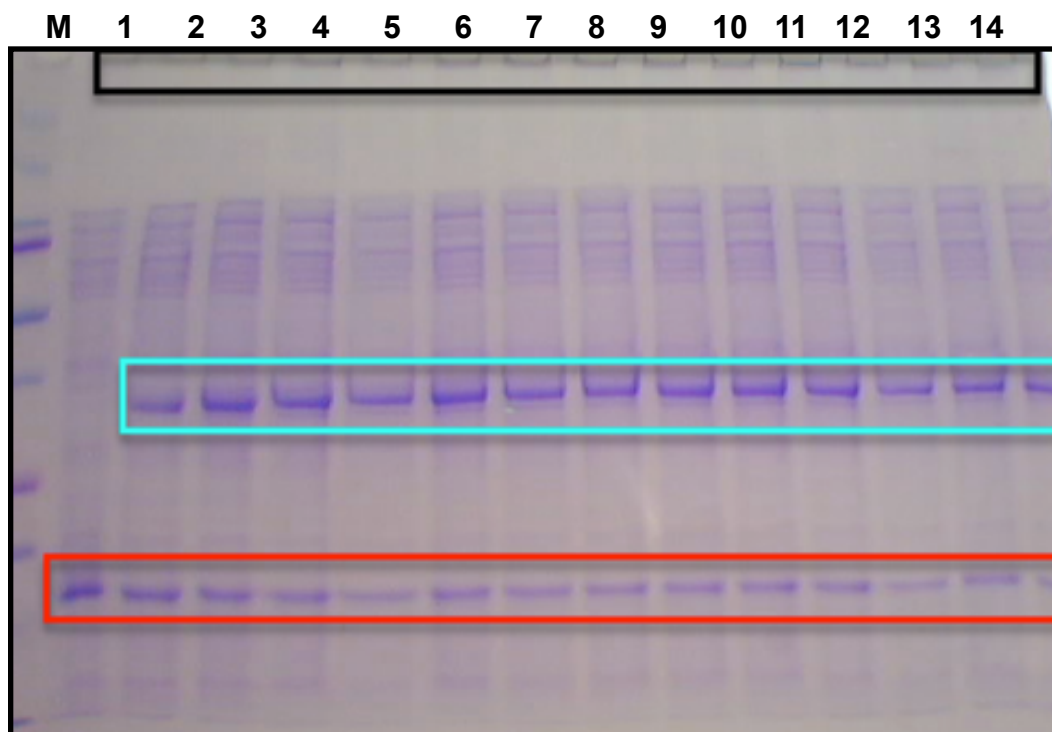


Figure 44. **Effect of decreasing the concentration of reducing agent on -7S with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

As can be seen in the above gel, there is no evidence for protein that does not enter the gel (the black box) but rather the protein is present as mostly di-mers (cyan box) with some monomers as well (red box). The model shown below would be consistent with these results.

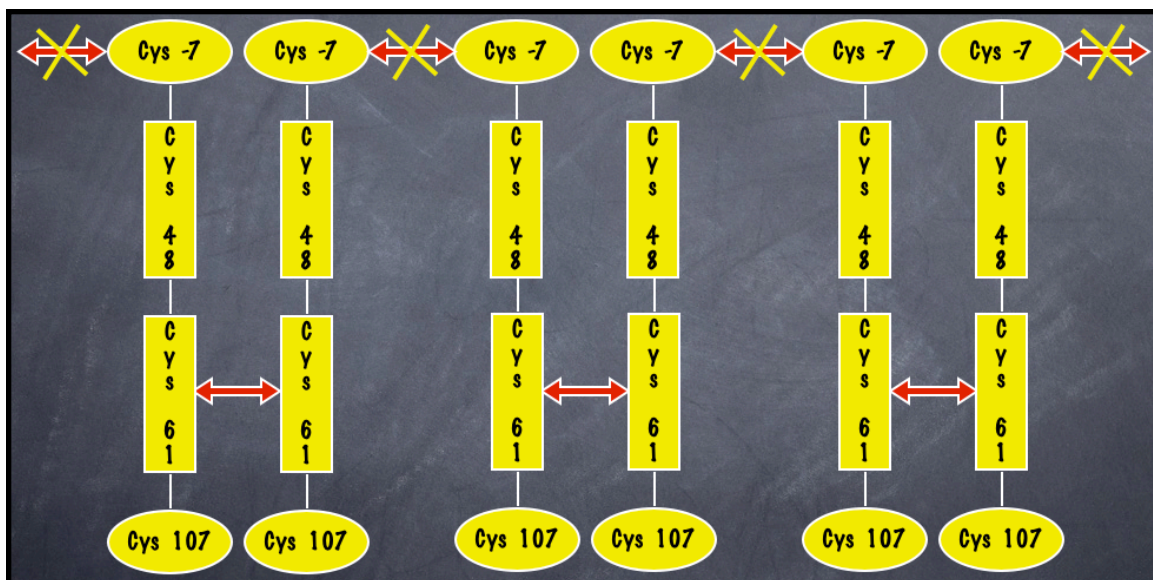


Figure 45. **Inter-molecular disulfide bond formation observed from figure 44**

It can be observed from figure 44 that with decreasing concentration of reducing agent from left to right, monomers as well as di-mers are formed. The di-mers (the band at the center of the gel) are possible due to inter-molecular disulfide bond formation between the cysteine at 61 of identical monomers (7). Some of them might not form the inter-molecular disulfide bonds leading to the formation of monomers (The band at the bottom of the gel). Moreover, there is no protein observed on top of the wells as could be seen in wild-type recombinant HBeAg. This must be because the cysteine at -7, which is responsible for formation of inter chain disulfide bonds between dimers has been replaced by serine.

-7S A and -7S B:

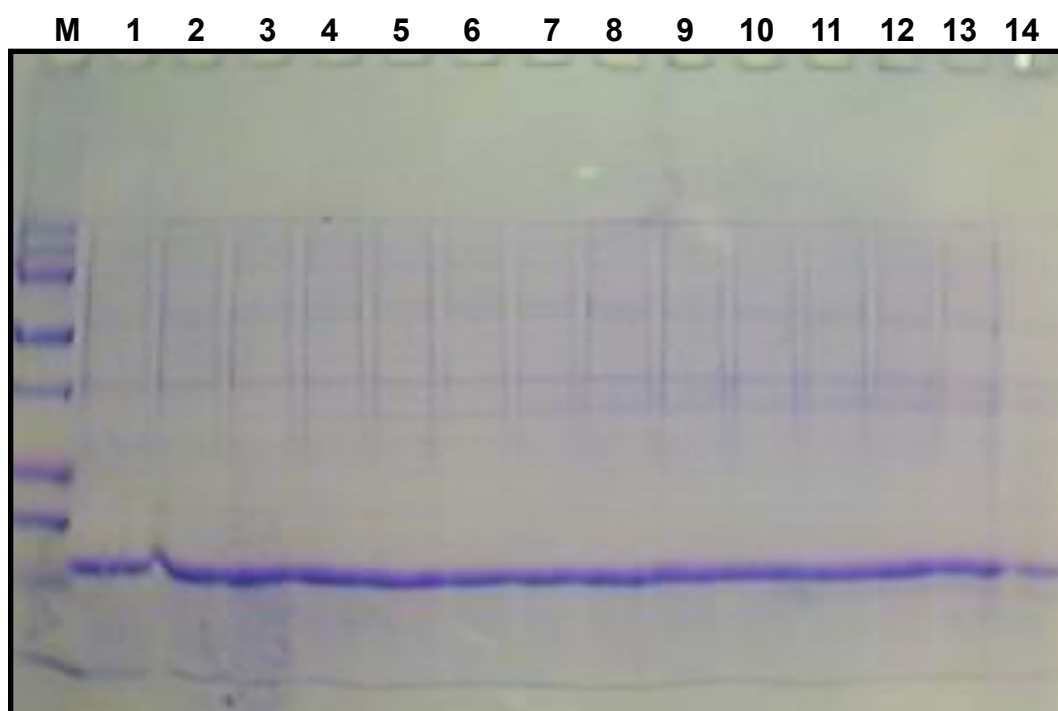


Figure 46. **Effect of decreasing the concentration of reducing agent on -7S A without iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

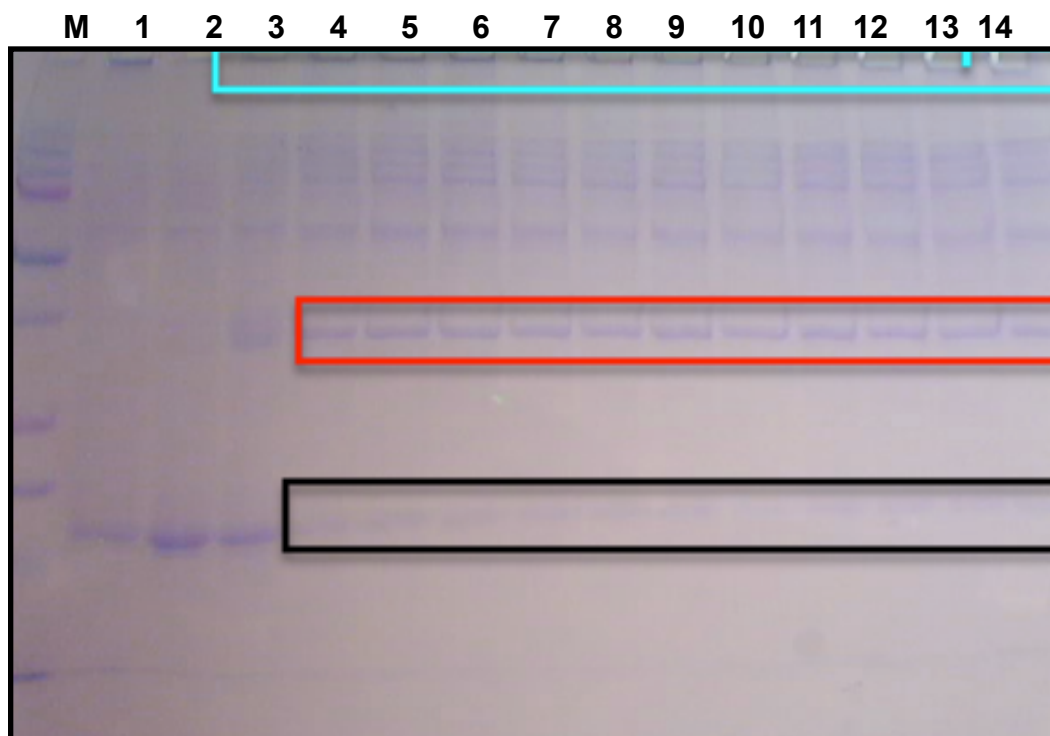


Figure 47. **Effect of decreasing the concentration of reducing agent on -7S A with iodoacetamide. treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

As can be seen in the above gel, there is no evidence for protein that does not enter the gel (cyan box) but rather the protein is present as mostly di-mers (red box) with very few monomers as well (black box). The model shown below would be consistent with these results.

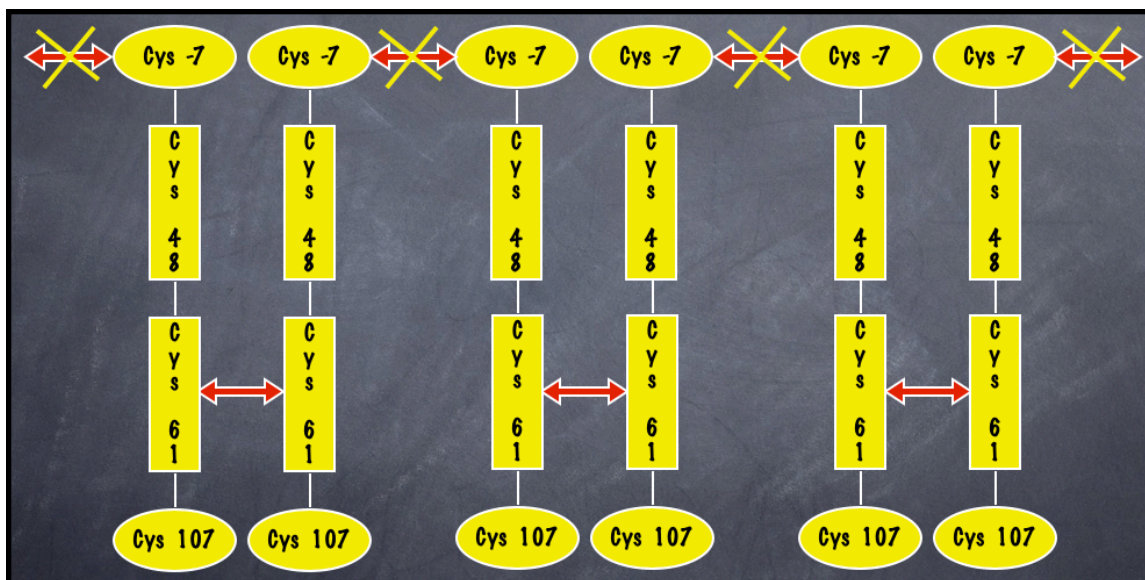


Figure 48. Inter-molecular disulfide bond formation observed from figure 47

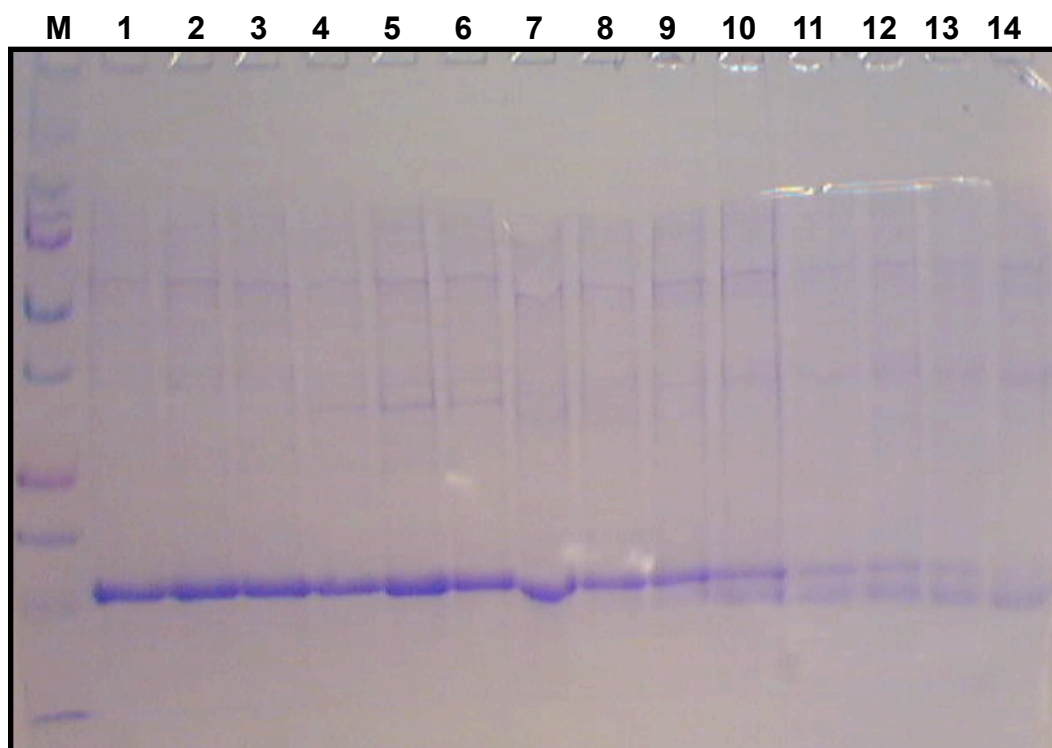


Figure 49. **Effect of decreasing the concentration of reducing agent on -7S B without iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

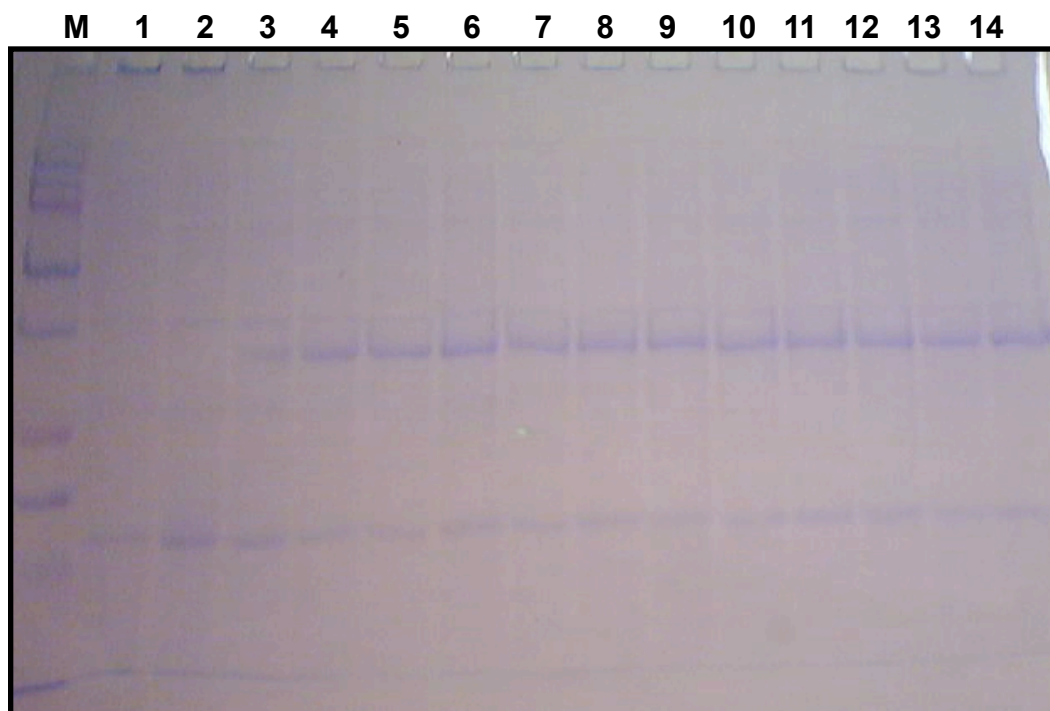


Figure 50. **Effect of decreasing the concentration of reducing agent on -7S B with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

From the above gel, the same is the conclusion as obtained in mutant -7S A. There is no evidence of protein not entering the gel and moreover there is formation of di-mers as well as very few monomers.

The following is the model shown bellow which is consistent with the results.

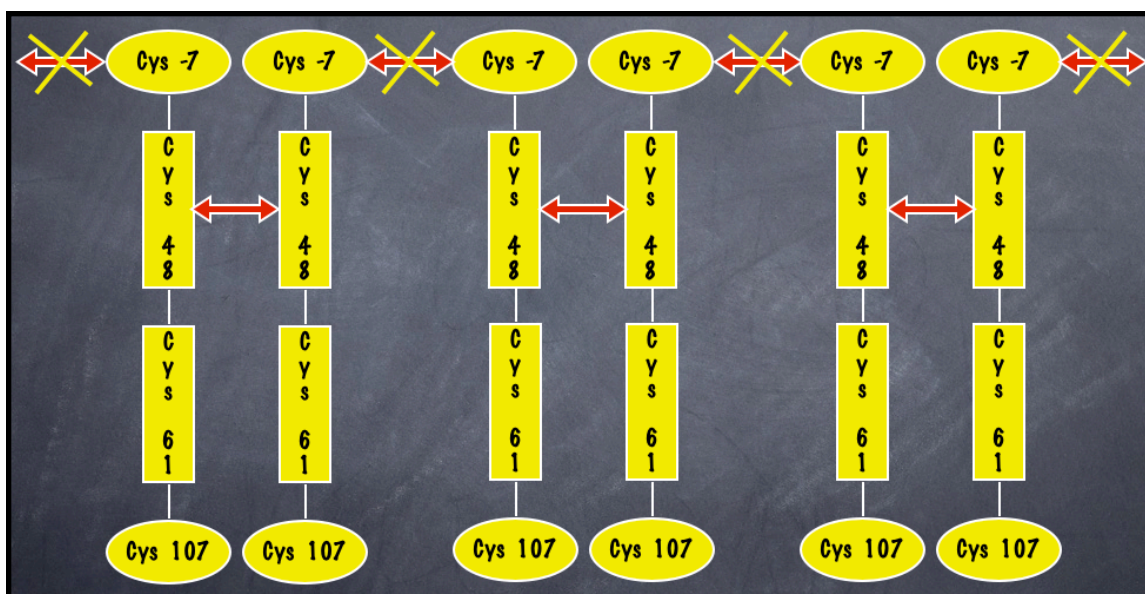


Figure 51. **Inter-molecular disulfide bond formation observed from figure 50**

It can be observed from figures 47 and 50 that with the decreasing concentration of reducing agent from left to right, there is majorly di-mer formation and very little monomer formation. The di-mer formation in figure 47 is due to inter-molecular disulfide linkage between cysteine at 61 of two identical monomers, since the cysteine at 48 is mutated in this mutant. Whereas, the di-mer formation in figure 50 is due to inter-molecular disulfide linkage between cysteine at 48 of two identical monomers, since the cysteine at 61 is mutated in this mutant. These are the same results as obtained by Jian Zheng and his group (5). Though they got these results on truncated HBcAg, the sequence for the truncated HBcAg and HBeAg is almost identical. Moreover, the cysteine at -7 is mutated in both the mutants and so no staining is observed on the top of the wells in both figures 47 as well as 50. This indicated that there are no high molecular weight cross-linked aggregates formed.

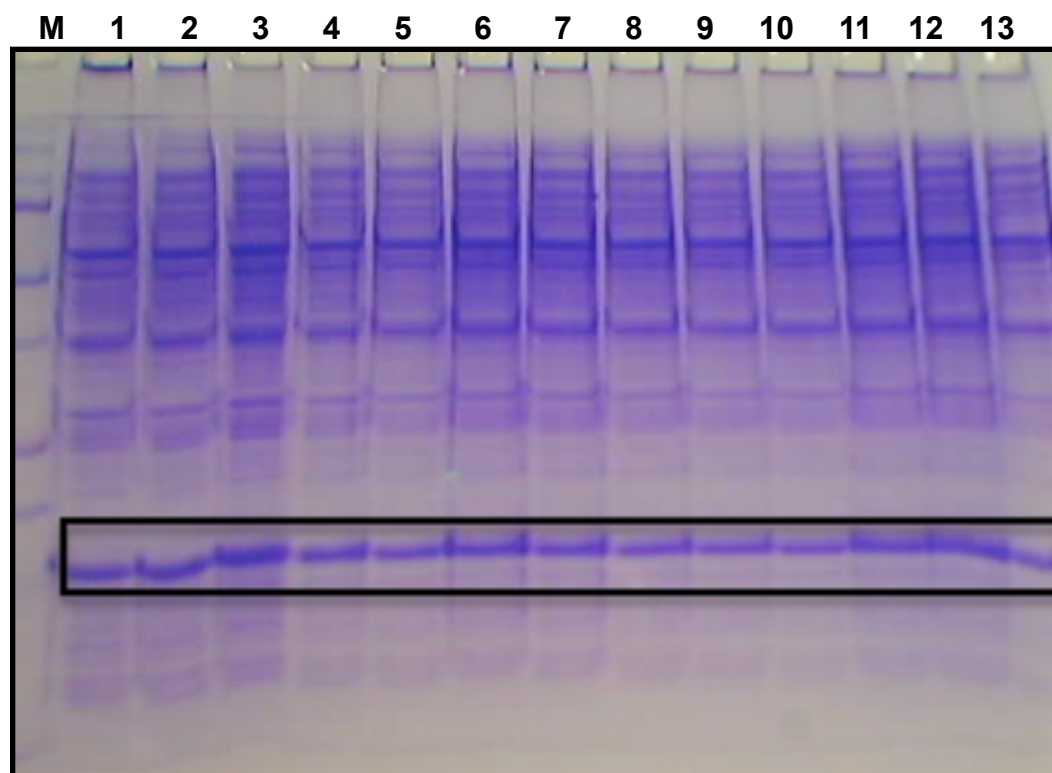
-7S C and PCC:

Figure 52. **Effect of decreasing the concentration of reducing agent on -7S C with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

The results obtained in the above mutant -7S C were not consistent with our hypothesis as we just saw formation of monomers (black box). We expected to see formation of di-mers as the concentration of reducing agent decreased but that did not occur. Thus, the -7S C did not give consistent results.

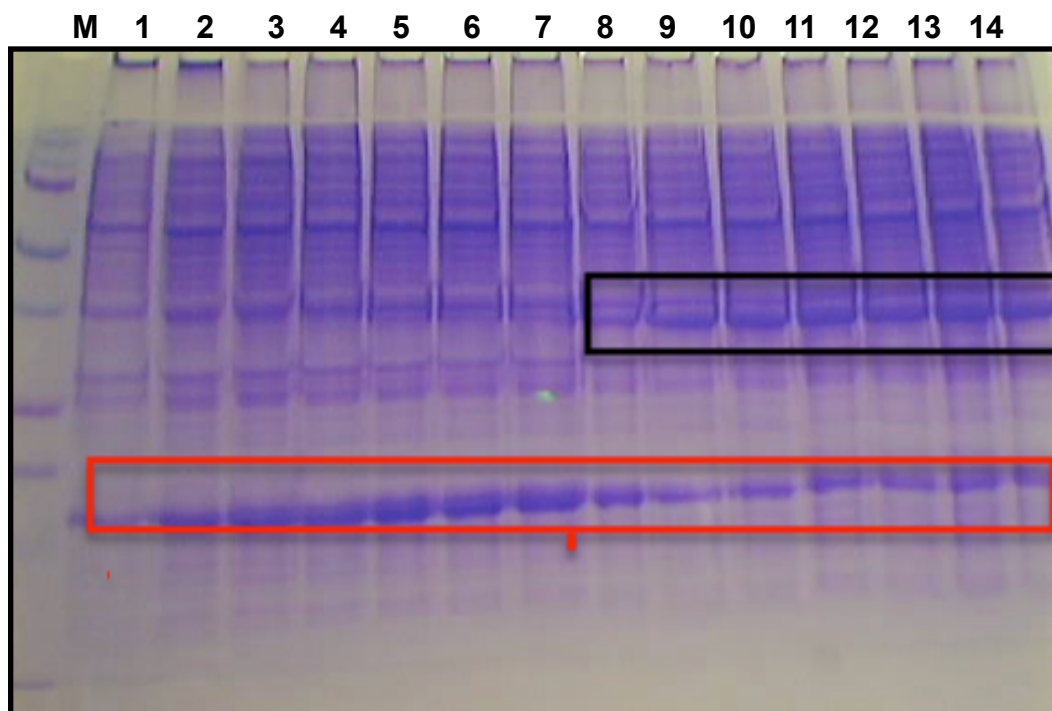


Figure 53. **Effect of decreasing the concentration of reducing agent on PCC with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

As can be seen from the above gel, the results obtained were consistent for this mutant. We saw di-mer formation (black box) and formation of monomers (red box) whereas formation of the fast moving band disappeared suggesting that the intra-molecular bond is between the cysteine at -7 and the cysteine at 107.

The following are the models consistent with the results.

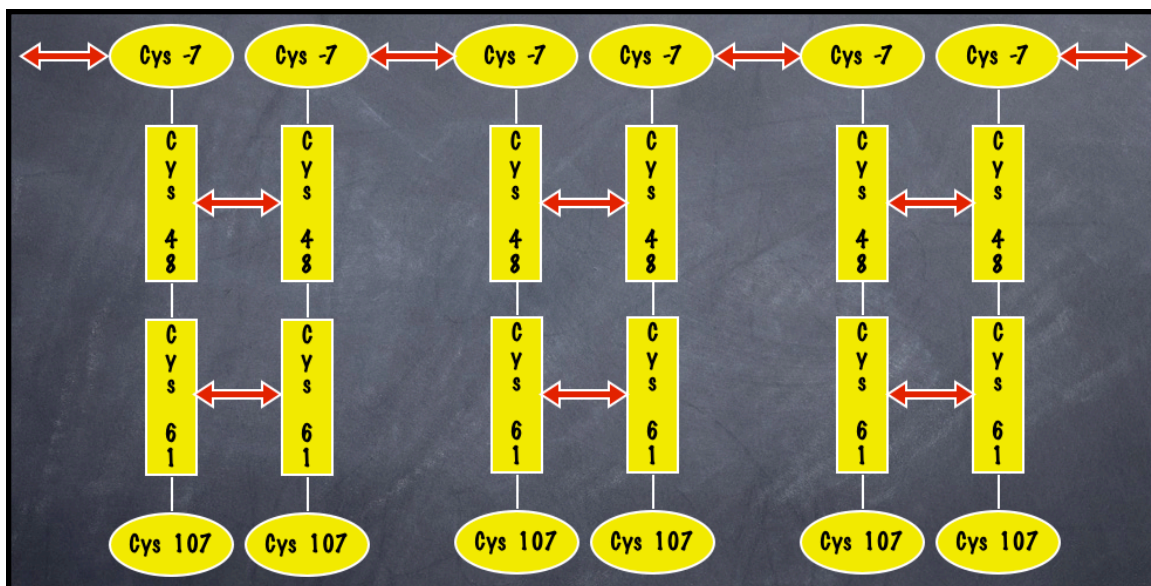


Figure 54. Inter-molecular disulfide bond formation observed from figure 53.

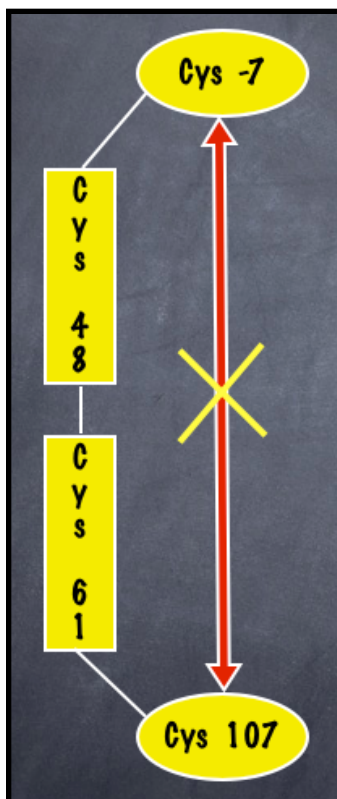


Figure 55. Disulfide bond formation observed from figure 53.

The results obtained in figure 52 were not as expected. We expected -7S C to give the same results as the PCC mutant. The PCC mutant just had the cysteine at 107 mutated whereas the -7S C had the cysteine at -7 mutated along with the cysteine at 107. Thus, both the mutants would prevent formation of the intra-molecular disulfide bonds. We still expected the presence of di-mers as the concentration of reducing agent decreased because the cysteine at 48 and 61 were present. But we only obtained monomers for the -7S C mutant. So this was not consistent with what results we had obtained so far.

Moreover, figure 53 gave consistent results. As the concentration of reducing agent was decreased from left to right, di-mer formation was observed along with formation of monomers but the formation of the band due to intra-molecular disulfide bond (from result below) had disappeared thus, indicating that cysteine at 107 was involved in making intra-molecular disulfide bonds with cysteine at position -7. Furthermore, the di-mers were still formed since both cysteine at 48 and cysteine at 61 were present in this mutant.

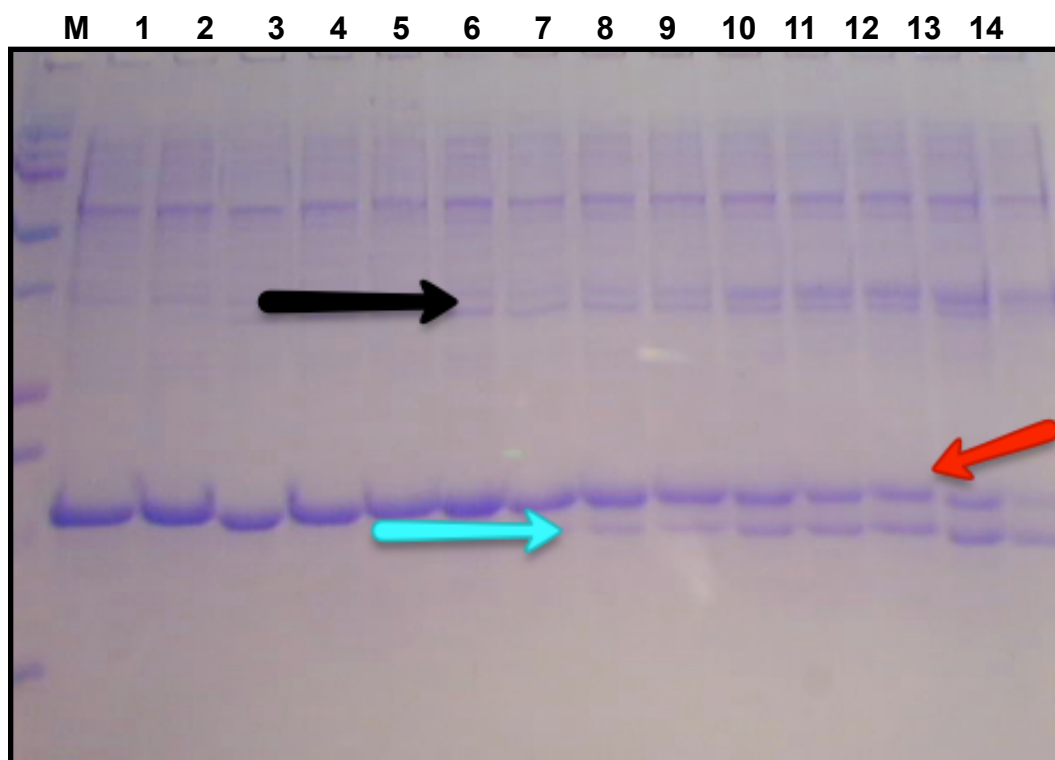
PCA and PCB:

Figure 56. **Effect of decreasing the concentration of reducing agent on PCA without iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

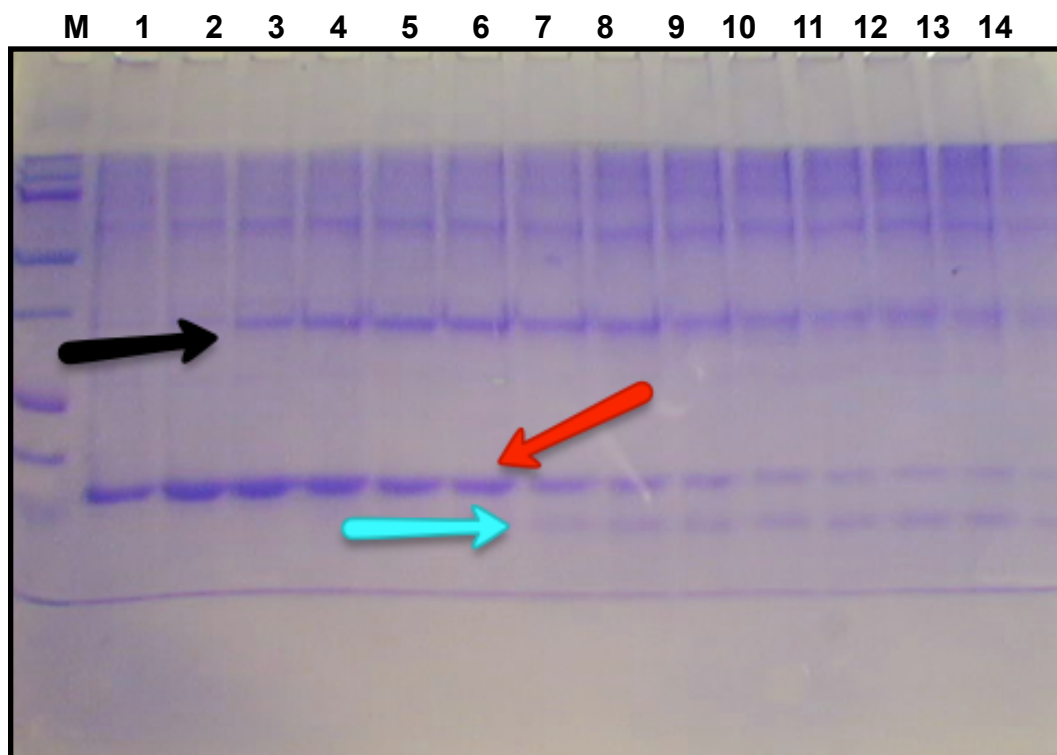


Figure 57. **Effect of decreasing the concentration of reducing agent on PCA with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

As can be seen from the above gels, there is di-mer formation (black arrow) along with monomer formation (red arrow). But here there is also the formation of the fast moving band (cyan arrow) which is caused due to intra-molecular disulfide bond formation.

The following are the models that are consistent to the results.

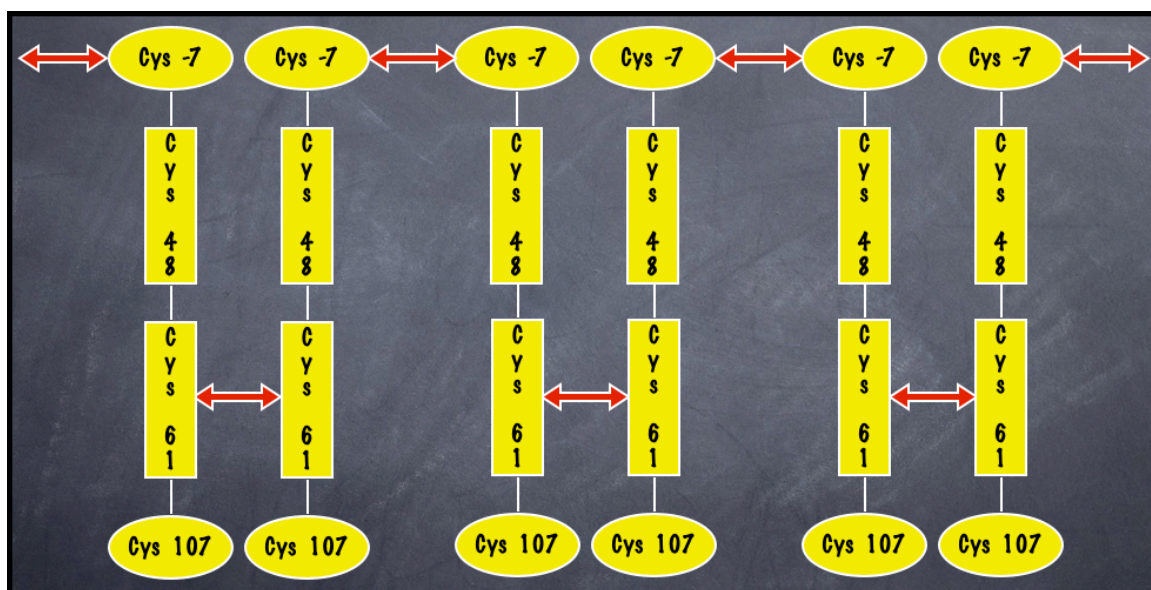


Figure 58. Inter-molecular disulfide bond formation observed from figure 57.

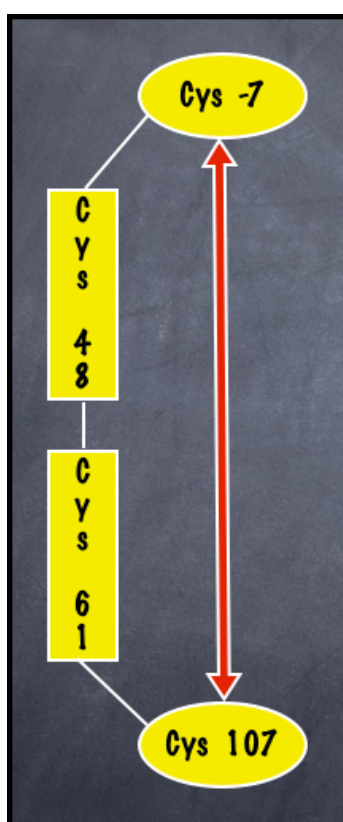


Figure 59. Intra-molecular disulfide bond formation observed from figure 57.

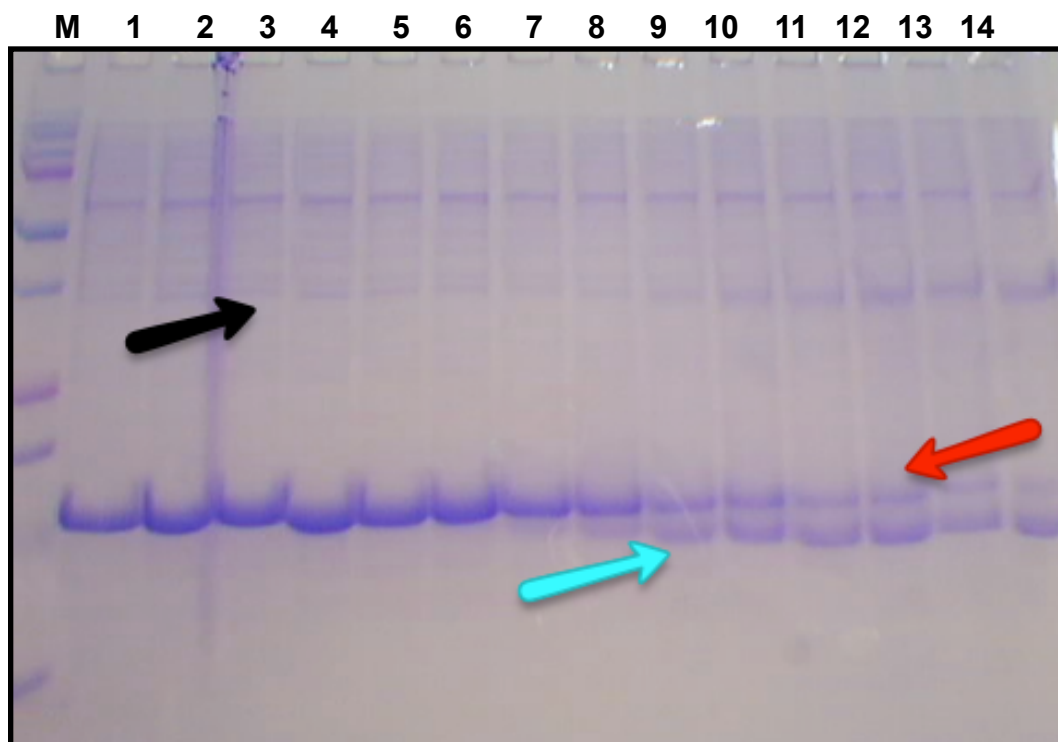


Figure 60. **Effect of decreasing the concentration of reducing agent on PCB without iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

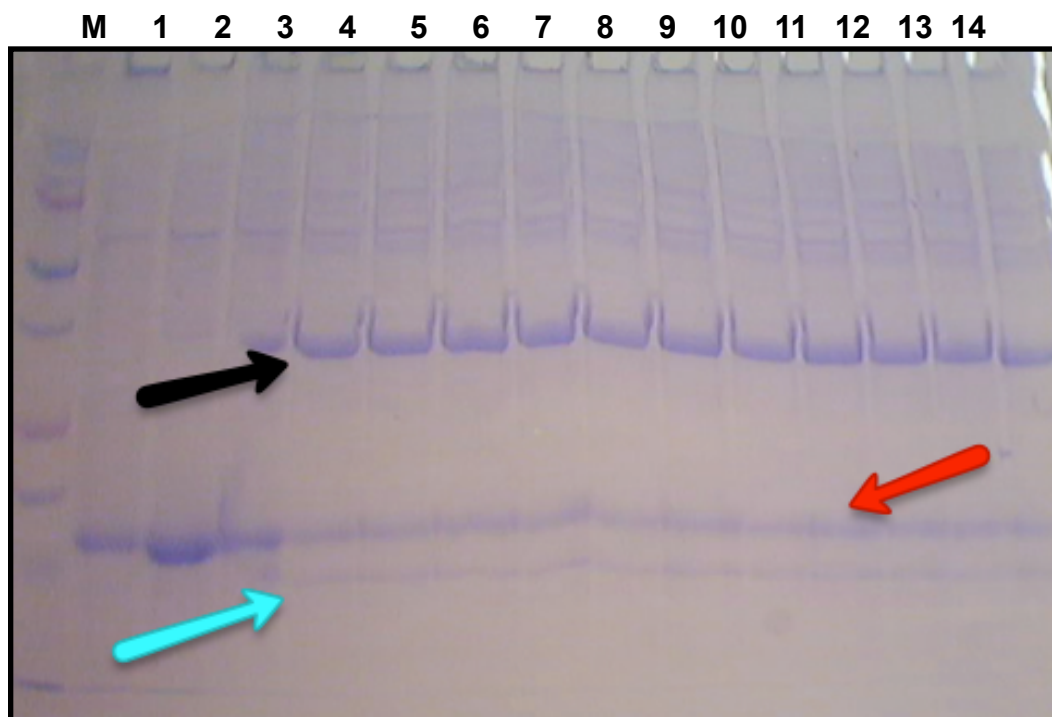


Figure 61. **Effect of decreasing the concentration of reducing agent on PCB with iodoacetamide treatment.** Lane 1 contains the highest amount of reducing agent. The reducing agent in the following lanes is then serially diluted and the lane 14 contains no reducing agent.

As can be observed from the above gels, the results are consistent with the PCA mutant. There is formation of di-mers (black arrow) along with formation of monomers (red arrow) and also the fast moving band (cyan arrow) is seen.

The following are the models consistent to the results.

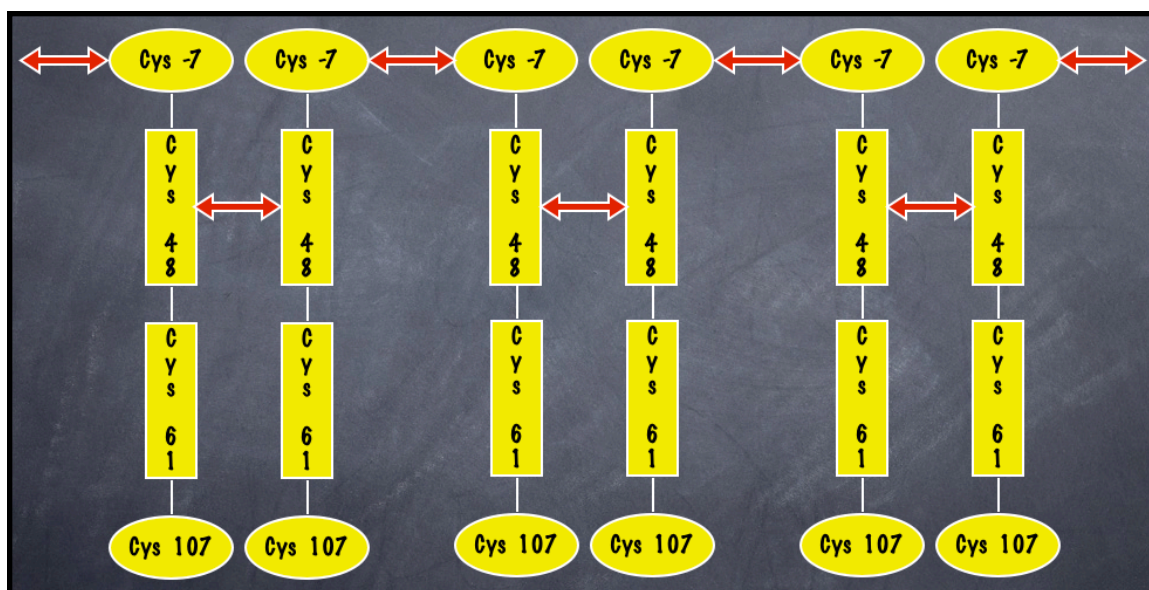


Figure 62. Inter-molecular disulfide bond formation observed from figure 61.

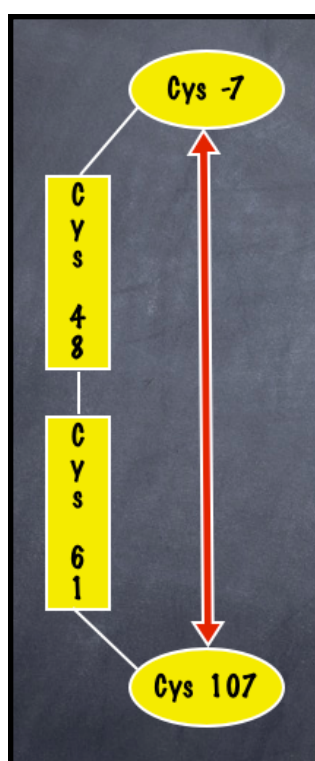


Figure 63. Intra-molecular disulfide bond formation observed from figure 61.

It can be observed from figures 57 and 61 that with decreasing concentrations of reducing agent from left to right, there is formation of di-mers as well as monomers. The important part to be observed here is the presence of two bands at the bottom of the gel. The second band from the bottom is due to monomers whereas, the first band from the bottom might be due to intra-molecular disulfide bond formation as mentioned by Wasenauer and group (7). According to the results obtained from figure 57, the intra-molecular disulfide bond is between the cysteine at position -7 and the cysteine at position 107. This can be predicted because in both the mutants, there has been no mutations at position cysteine -7 and cysteine 107. Moreover, in mutants with mutation at cysteine -7 and cysteine 107, these bottom bands were not obtained. Further, the dimer formation here is again due to inter-molecular disulfide bond formation between the cysteine at 61 and cysteine at 48 of two identical monomers.

Quantitation of Sulfhydryl Groups

The standard cysteine curve was first plotted to check if the line passed through zero and gave a linear relationship between absorbance and cysteine concentration.

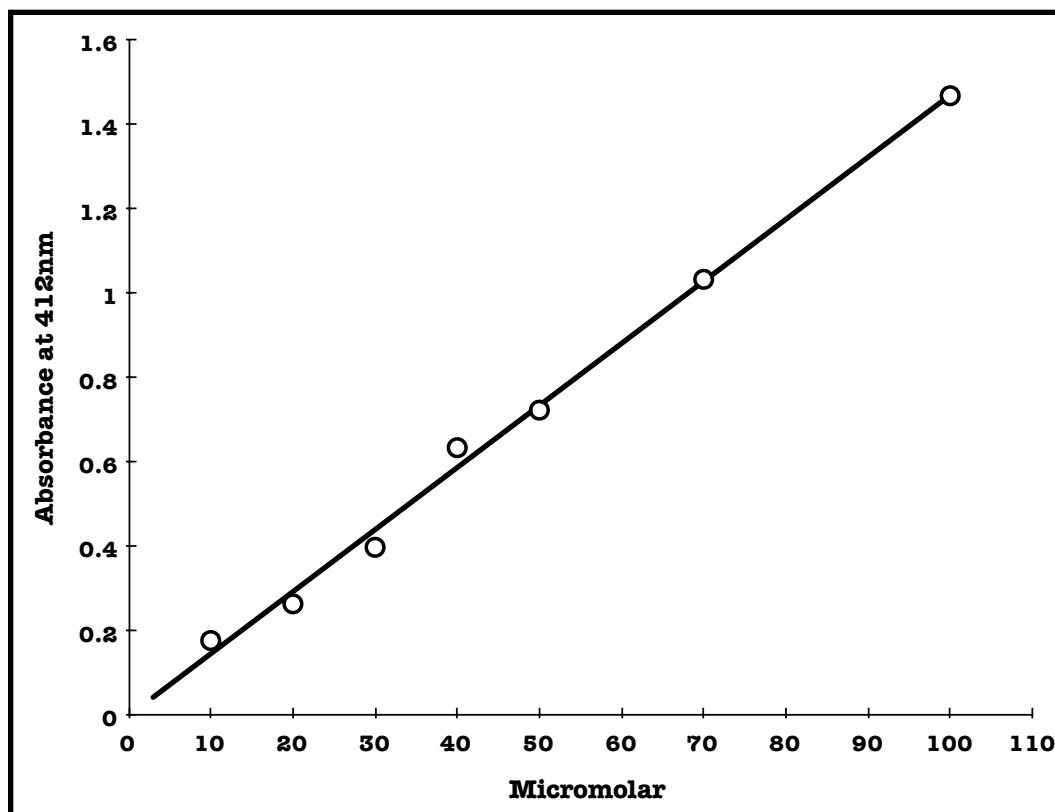


Figure 64. **Cysteine Standard Curve.** The tubes containing 10 μ M, 20 μ M, 30 μ M, 40 μ M, 50 μ M, 70 μ M and 100 μ M of cysteine stock solution were reacted with Ellman's reagent and their absorbance readings were taken at 412nm. These values were plotted to obtain the cysteine standard curve.

The following is the table showing the absorbances obtained at 412nm and the moles of -SH/Mole of protein for all the mutants.

	SDS Treated	Moles of -SH/ Mole of Protein	Non - SDS	Moles of -SH/ Mole of Protein
HBeAg	0.333	0.5	0.231	0.3
-7S	0.206	0.3	0.151	0.25
-7S A	0.256	0.5	0.221	0.4
-7S B	0.344	0.57	0.190	0.3
-7S C	0.351	0.6	0.341	0.55
PCA	0.310	0.5	0.184	0.25
PCB	0.346	0.57	0.153	0.25
PCC	0.367	0.57	0.326	0.55

Table 3. **Spectrophotometer readings at 412nm along with the moles of -SH/ Mole of proteins.**

From the above values for the native as well as the mutant proteins, it was observed that approximately 0.5 cysteine residues were always present as free sulfhydryl. Jian Zheng and his group had stated that the cysteine at 107 was always free in truncated HBcAg (5). The sequence of the truncated HBcAg is almost identical to HBeAg. At first on treating the HBeAg with Ellman's reagent, we obtained almost no free cysteine for most of the mutants, but on treating the sample with 0.1% SDS, half cysteine was obtained as free. This half cysteine might be due to the cysteine at 107 which is sometimes free and sometimes is involved in an intra-molecular disulfide bond with cysteine at -7 or may be due to several of the cysteines being partially unpaired. In

any case, since the protein has 4 cysteines it is clear that they are almost all involved in disulfide bonding. Moreover, since all mutations do not increase the content of free cysteine, each of the cysteines must pair only between identical residues (-7 to -7, 48 to 48, 61 to 61). If, for example cysteine 48 were disulfide bonded to cysteine 61, then mutation of cysteine 48 would lead to a free sulfhydryl for cysteine 61. Since the samples had to be treated with 0.1% SDS to react maximally, this suggests that some of the cysteines are buried and therefore are not able to react with the Ellman's reagent. Also, from previous research work (5) regarding core structure it can be said that the cysteine at 48 is also not always involved in making inter-molecular disulfide bonds between cysteine at 48 of two identical monomers. Therefore, we can conclude from mutants -7S, -7S B, -7S C, PCB and PCC that in HBeAg, the half free cysteine may sometimes also be due to the cysteine at 48 which might not always be involved in inter-molecular disulfide bonding. This is because all the mutants have the Cys at 48 unmutated.

To further check if the cysteine disulfide bonds were exposed on surface or not for reduction, the ReduceIM column was used. It was observed that on passing the protein through this column, it was not reduced indicating that all the cysteine disulfide bonds were buried and none of them were exposed on the surface (Results not shown).

Circular Dichroism Spectra of the Recombinant HBeAg and its Mutants

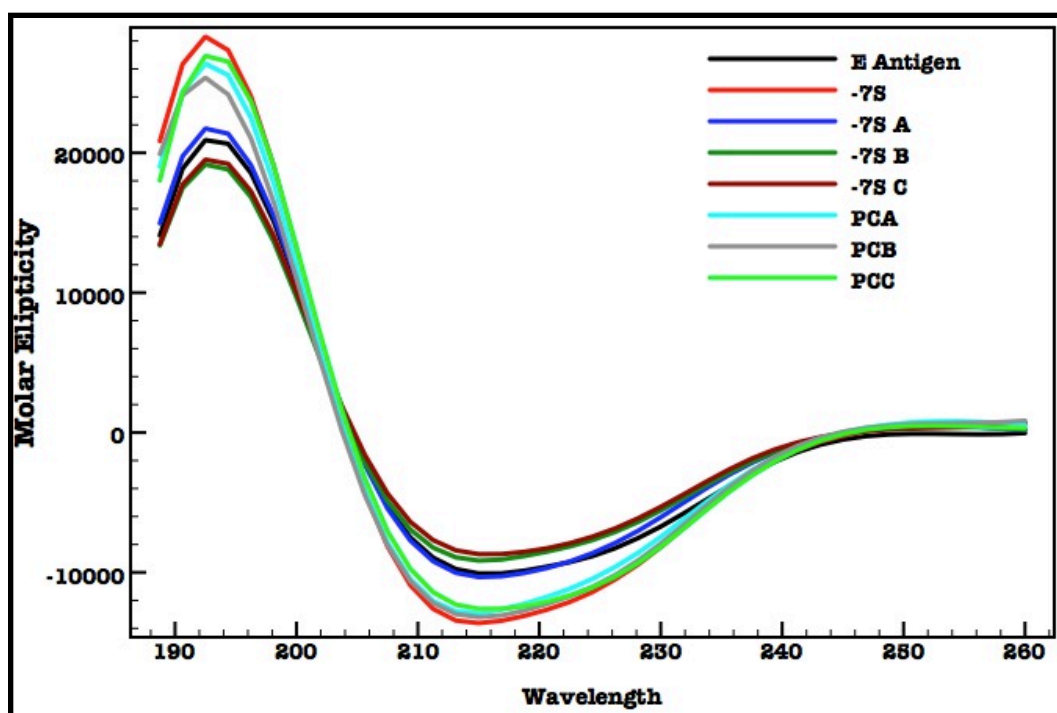


Figure 65. **Circular dichroism of all the proteins.**

The circular dichroism spectra of each of the proteins was determined. Since these proteins differ only by conservative point mutations and because the proteins behaved identically during purification, we were not expecting to find any gross differences in the secondary structures between the mutants and the wild type. However, from the data we obtained in figure 65, we cannot rule out that there are some differences. The spectra appeared to fall into two groups that had some differences in the secondary structures between them. The E antigen, -7S A, -7S B and -7S C was one group that had almost similar secondary structures and on the other hand -7S, PCA, PCB and PCC was the other group that had almost similar secondary structures. However, due to differences in purity and difficulty in quantitating the protein

concentration due to differences in the A280 caused by light scattering, we would not conclude that these differences are real.

Light Scattering

Dynamic light scattering measurements were made on each of the proteins to examine the molecular size. The following are the results obtained. From the results it can be concluded that all of the proteins assemble into a particulate form, although the sizes do vary. The particle sizes ranged from 30nm - 43nm.

The following are the results obtained for wild-type recombinant HBeAg.

Protein Solutions

14E6A.EXP

Annotations

New Experiment

Sep 08 11:45 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	66	38.29	114402.5	15.1	39.3	0.16
Cumu. Bimodal 1	105	25.46	39109.1	-	-	-
Cumu. Bimodal 2	38	92.52	8360233.7	-	-	-
Regularization 1	308	8.22	1124.0	-	-	-
Regularization 2	74	38.44	115075.3	-	-	-

Figure 66. Light scatter data showing particle size of wild-type recombinant HBeAg.

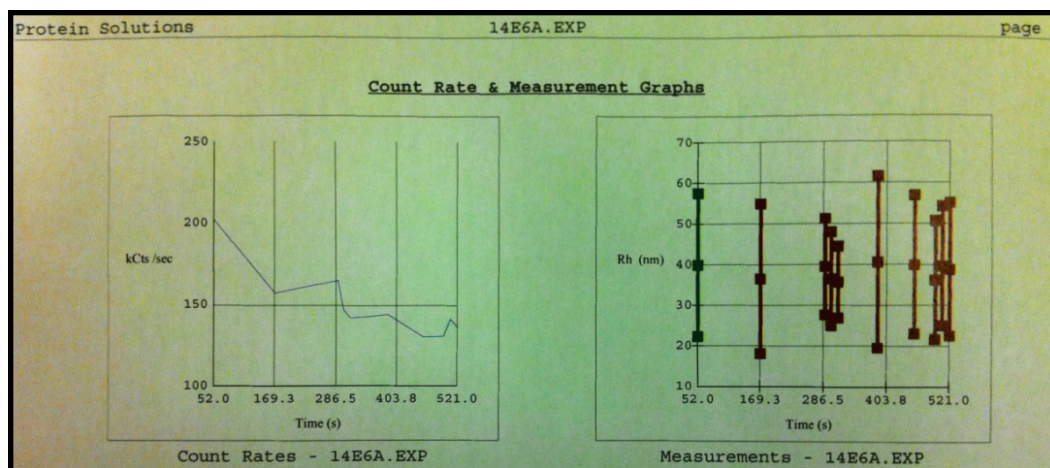


Figure 67. Graph showing 10 data points obtained for wild-type recombinant HBeAg

The following are the results obtained for -7S.

Protein Solutions

-7S.EXP

Annotations

New Experiment

Sep 08 12:28 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	90	30.23	56650.1	14.6	47.9	0.24
Cumu. Bimodal 1	130	21.25	20349.3	-	-	-
Cumu. Bimodal 2	46	60.67	498639.4	-	-	-
Regularization 1	110	31.69	64433.5	-	-	-
Regularization 2	18	156.60	7778141.0	-	-	-

Figure 68. Light scatter data showing particle size of -7S.

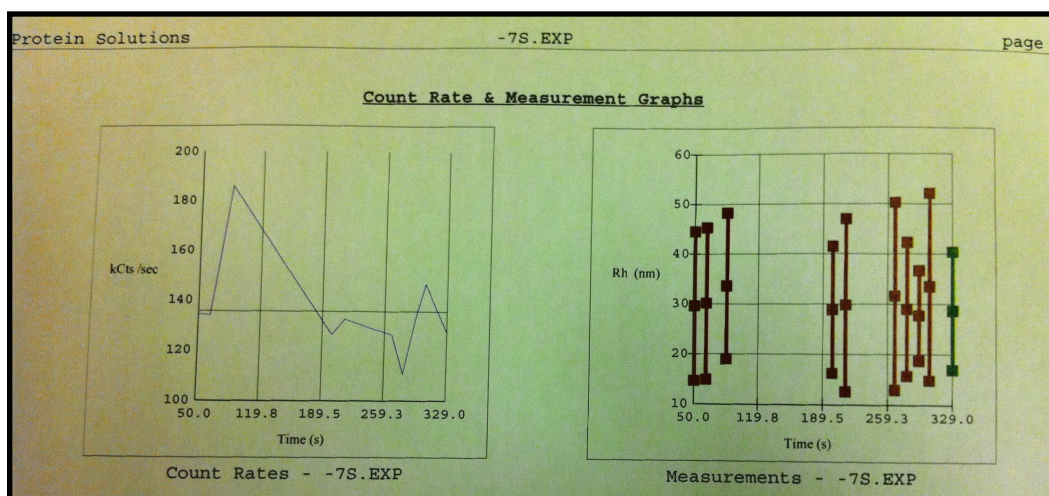


Figure 69. Graph showing 10 data points obtained for -7S.

The following are the results obtained for -7S A.

Protein Solutions

-7SA.EXP

Annotations

New Experiment

Sep 08 14:02 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	62	43.67	169670.7	21.2	48.6	0.24
Cumu. Bimodal 1	115	23.97	29293.9	-	-	-
Cumu. Bimodal 2	37	73.93	864135.0	-	-	-
Regularization 1	308	8.82	1391.5	-	-	-
Regularization 2	71	52.48	292731.5	-	-	-

Figure 70. Light scatter data showing particle size of -7S A.

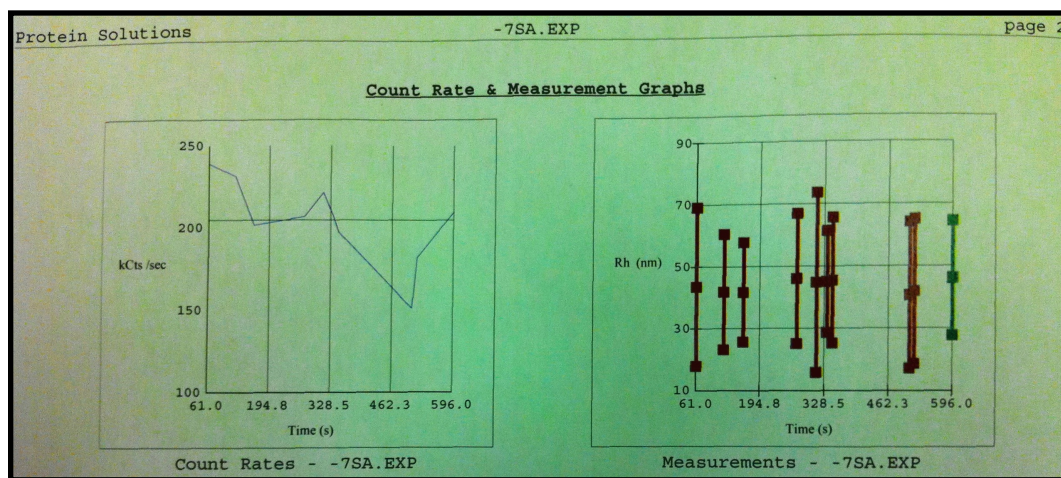


Figure 71. Graph showing 10 data points obtained for -7S A.

The following are the results obtained for -7S B.

Protein Solutions

-7SB.EXP

Annotations

New Experiment

Sep 08 13:15 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	73	37.17	104572.9	17.9	47.8	0.24
Cumu. Bimodal 1	124	23.12	28614.2	-	-	-
Cumu. Bimodal 2	42	74.25	1524047.7	-	-	-
Regularization 1	84	46.04	197674.9	-	-	-

Figure 72. Light scatter data showing particle size of -7S B.

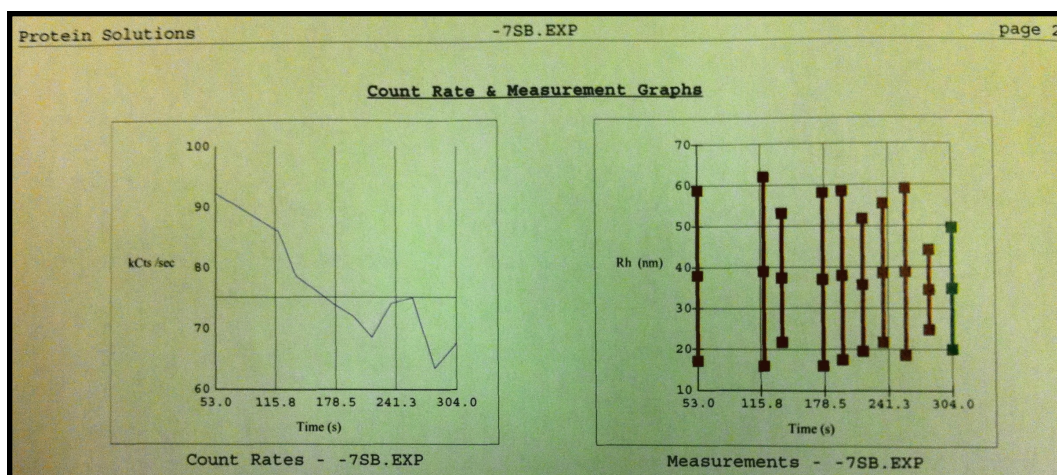


Figure 73. Graph showing 10 data points obtained for -7S B.

The following are the results obtained for -7S C.

Protein Solutions

7SC.EXP

Annotations

New Experiment

Sep 20 15:56 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	81	31.53	64233.4	14.0	44.4	0.20
Cumu. Bimodal 1	126	20.84	20105.0	-	-	-
Cumu. Bimodal 2	48	58.26	560124.4	-	-	-
Regularization 1	308	8.29	1154.0	-	-	-
Regularization 2	93	32.51	69583.5	-	-	-

Figure 74. Light scatter data showing particle size of -7S C.

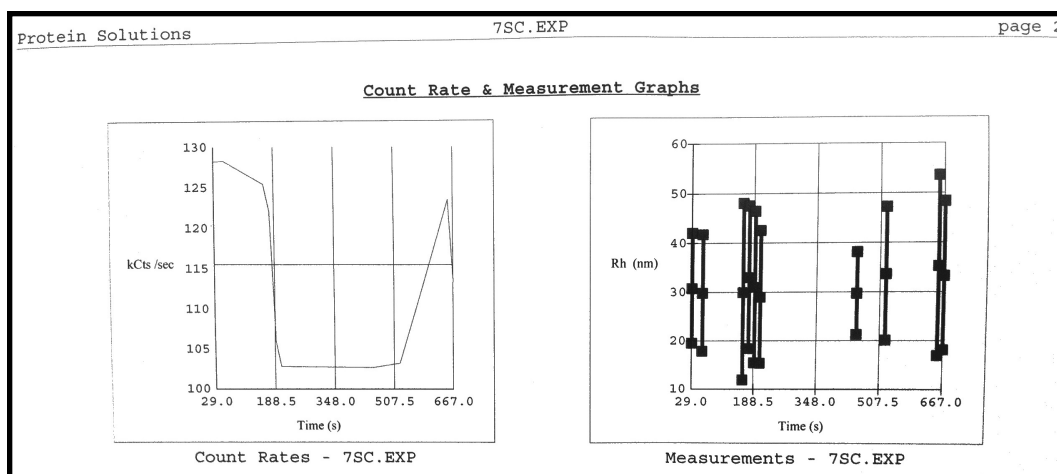


Figure 75. Graph showing 10 data points obtained for -7S C.

The following are the results obtained for PCA.

Protein Solutions		PCA.EXP				
<u>Annotations</u>						
New Experiment						
Sep 08 13:29 2010 (Dynamics v4.0)						
<u>Results Summary</u>						
	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	64	42.41	156856.5	20.3	47.9	0.23
Cumu. Bimodal 1	116	24.09	30107.0	-	-	-
Cumu. Bimodal 2	40	69.03	726830.6	-	-	-
Regularization 1	436	6.25	494.7	-	-	-
Regularization 2	70	54.58	329315.5	-	-	-

Figure 76. Light scatter data showing particle size of PCA.

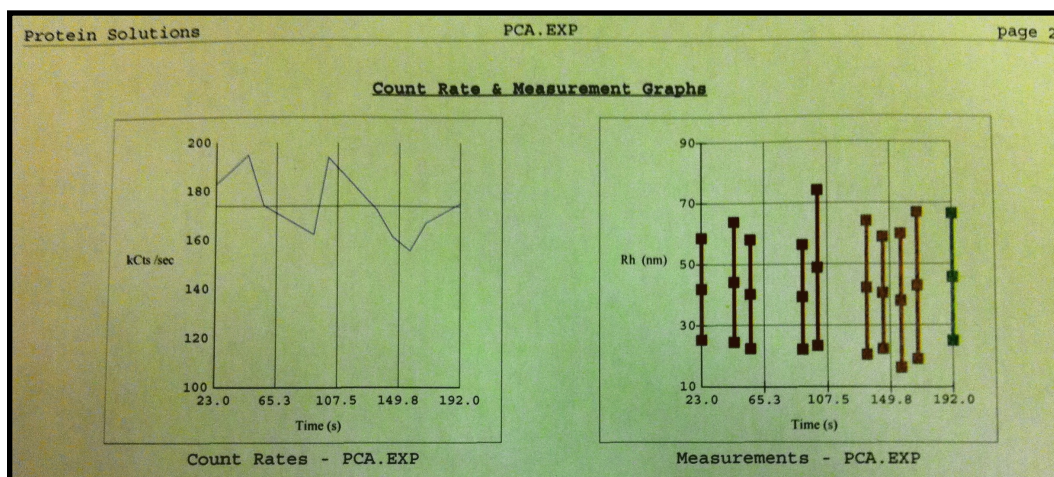


Figure 77. Graph showing 10 data points obtained for PCA.

The following are the results obtained for PCB.

Protein Solutions

PCB.EXP

Annotations

New Experiment

Sep 08 13:39 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	67	40.37	134420.3	18.3	45.3	0.21
Cumu. Bimodal 1	129	22.71	27880.4	-	-	-
Cumu. Bimodal 2	42	66.98	734065.0	-	-	-
Regularization 1	602	4.51	186.3	-	-	-
Regularization 2	74	52.62	295087.3	-	-	-

Figure 78. Light scatter data showing particle size of PCB.

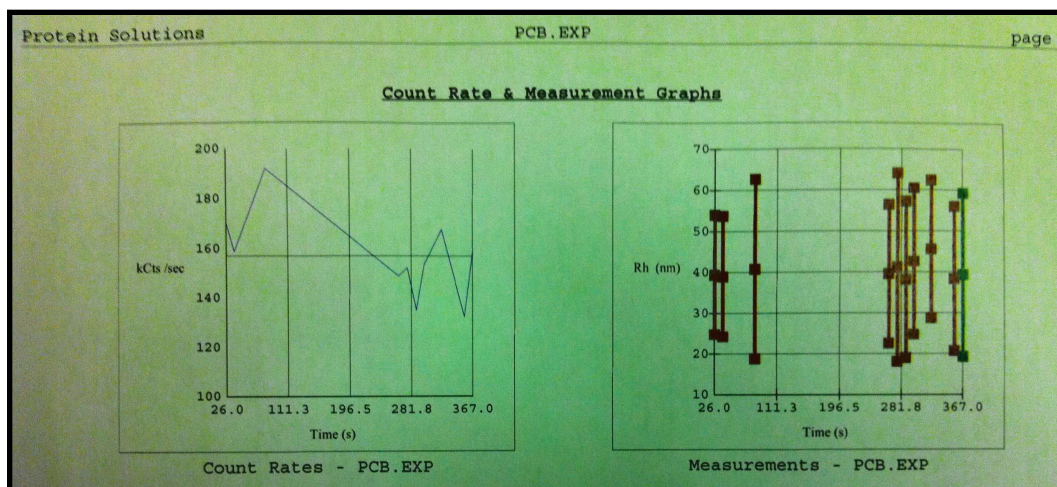


Figure 79. Graph showing 10 data points obtained for PCB.

The following are the results obtained for PCC.

Protein Solutions

PCC1.EXP

Annotations

New Experiment

Sep 20 15:33 2010 (Dynamics v4.0)

Results Summary

	Dt (1e-9*cm/s^2)	Rh (nm)	MW (kDa)	Cp (nm)	Cp/Rh (%)	Cp/Rh Index
Cumu. Monomodal	82	30.08	55606.3	9.8	32.3	0.11
Cumu. Bimodal 1	109	23.58	28776.7	-	-	-
Cumu. Bimodal 2	39	93.82	6487011.4	-	-	-
Regularization 1	98	28.09	44877.1	-	-	-

Figure 80. Light scatter data showing particle size of PCC.

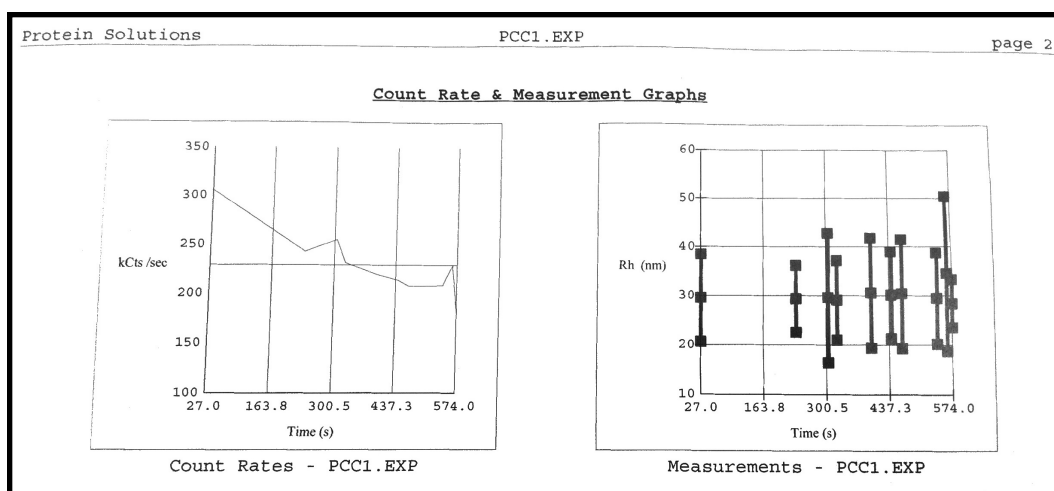


Figure 81. Graph showing 10 data points obtained for PCC.

Chapter 6: *Conclusions*

At the outset, it can be noted from our data that the expression levels of the wild type as well as the mutants varied. The variations were not on a large scale but one would not expect such variations in expression levels since the only change between the mutants was a cysteine substituted to a serine. Further, the purity levels also varied due to the differences in expression levels, but for most part, these variations in concentration and purity between the mutants did not affect our conclusions.

The ELISA data showed that all the mutants as well as the wild type had high antigenicity. The antigenicity did vary somewhat, and this could be due to the variation in concentrations of the mutants. This was because the absorbance at 280 nm was used to determine the concentrations of all the proteins. It is important to note here that the percentage purity of all the proteins was not the same and so when the absorbance at 280nm was taken for the proteins, the impurities present along with the proteins also got measured. Thus, the concentrations obtained were not only the concentration of our protein but it also included the other proteins (impurities) which were present in the solution in minute amounts. Due to this reason we saw some variations in the antigenicity but from the ELISA we can conclude that all the mutants did have antigenic activity.

Moreover, light scattering results obtained showed that all the proteins assembled in to particulate antigens and the size of all the proteins varied from 30 to 43 nanometers. This result is consistent with their elution from Sepharose CL4B, which has

an exclusion limit of about 10 million, and the antigen eluted after the void volume, but well before low molecular weight proteins were eluted.

The circular dichroism data showed that all the mutants had a high alpha helix content. There may be differences, but due to the differences in purity of the proteins, it was not possible to conclude that the differences observed in the CD spectra represent true differences. It could also be observed from the CD spectra of the mutants that the proteins fell into two groups that had some differences in the secondary structures between them. The E antigen, -7S A, -7S B and -7S C was one group that had almost similar CD spectra and on the other hand -7S, PCA, PCB and PCC was the other group that had almost similar CD spectra. Thus it seems as if the double mutant species may have differences from the single mutants. Before this conclusion could be verified, it would have to be shown that the purity of all the proteins was the same, and that the protein concentrations used were identical. This, as noted above presents some problems that have not yet been resolved.

It has been previously found that there are inter-molecular disulfide bonds in HBcAg. The cysteine at 61 was always and the cysteine at 48 was partly involved in inter-molecular disulfide bond formation with the identical residue of other monomer. The cysteine at 183 was also always involved in inter-molecular disulfide bond formation with the identical residue of other monomer (6). Moreover, it was also observed that in HBeAg the cysteine at 61 was always involved in an inter-molecular disulfide bond formation with the identical residue of the other monomer (7). Now from our studies, the 2D gel electrophoresis on the E antigen showed that the E antigen was present in a range from monomers to oligomers suggesting that there were multiple

inter-molecular disulfide bonds. Also, one species of protein was observed on this gel, that by its presence to the left of the diagonal, migrated faster under non-reducing conditions than under reducing conditions. This is what is expected for proteins that have intra-molecular disulfide bonds. From our results obtained by checking the effect of decreasing concentration of reducing agent of all the mutants on SDS PAGE gels it could be concluded that the cysteine at 61 was always and the cysteine at 48 was partly involved in inter-molecular disulfide bond formation with the identical residue of other monomer resulting in formation of di-mers. Unlike the previously reported results with the truncated HBcAg protein, very little of the wild-type HBeAg, under non-reducing conditions, even entered the stacking gel on SDS-PAGE. Thus the wild-type HBeAg must be present as a highly cross-linked polymer. However, on mutating the cysteine at -7, it was found that there were only presence of di-mers and monomers and the oligomers present on top of the wells which could not enter the gel had disappeared. Thus this mutant behaved identically to the truncated HBcAg. This leads us to conclude that the cysteine at -7 was involved in the formation of oligomers by forming disulfide bonds between the dimers, but that the dimers were held together by the same disulfide bonding pattern as had been found for the truncated HBcAg. Thus, it can be concluded that the di-mers formed by the inter-molecular disulfide bond formation by cysteine at 61 and 48 were further connected by the cysteine at -7 to form a lattice of protein oligomers of increasing sizes.

Further, from mutants PCA and PCB. a band was observed below the band observed for the monomers at the bottom of the gel, under non-reducing conditions. This band ran faster than the monomers on gel because of intra-molecular disulfide

bond formation. It has been previously concluded that there are intra-molecular disulfide bonds formed in HBeAg (7). It had later on been proposed that the intra-molecular disulfide bonds were between the cysteine at -7 and the cysteine at 61 (8). According to results obtained by us on mutant PCC (mutation of cysteine at 107), it could be concluded that the intra-molecular disulfide bond formation was between the cysteine at -7 and the cysteine at 107. Also, this intra-molecular disulfide bond formation was not present in all of the polypeptide chains, since the intensity of the band observed at this position was not very intense, thus corresponding to only a small population of this species of the protein.

It was further observed that all the disulfide bonds were buried and were not available on the surface for reduction. From the analysis using Ellman's reagent it was found that in HBeAg, approximately one-half of a cysteine was always present as the free sulfhydryl. It had been previously found that cysteine at position 107 was always free in HBcAg (6). We think this half free cysteine in HBeAg was either due to the cysteine at 107 which was sometimes free and not always involved in intra-molecular disulfide bonding or it was due to the cysteine at 48 which was sometimes free and not always involved in inter-molecular disulfide bonding.

Thus, from the data presented above we can conclude that the HBeAg was present in solution as a homogeneous particulate form of about 36 nm, while the mutants were of similar but not identical size. Although the particles were uniform in size, their disulfide bonding pattern differed in a consistent manner. For the wild-type HBeAg:

1. Small number of free monomers without any disulfide bonds, seen as monomers under non-reducing conditions.

2. Small number of monomers with formation of an intra-molecular disulfide bond between the cysteine at -7 and cysteine at 107, seen as more rapidly low molecular weight species that migrate more rapidly than monomers, but convert to monomers upon reduction.

3. Most of the monomers cross-linked into polymers of varying lengths in which di-mers were formed by the disulfide bonds between cysteines at 61 and 48, then subsequent disulfide bonding between different dimers connected through the cysteine at -7.

Chapter 7: *Future Directions*

The -7S C mutant did not give the results as expected. We expected that since the -7S C mutant had a mutation of the cysteine at position -7 as well as at position 107, there would be presence of dimers and the fast moving band due to intra-molecular disulfide bond formation would disappear. The formation of di-mers would be due to the presence of cysteine at position 48 and 61 whereas the fast moving band which is thought to be an intra-molecular disulfide bond formed between cysteine at -7 and cysteine at 107 would disappear since both of them were mutated in this mutant. But these are not the results what we obtained. We just obtained monomers as the concentration of reducing agent decreased on an SDS PAGE gel. These results obtained are not consistent with our hypothesis and therefore, further analysis will have to be done to perfectly identify the disulfide bonding pattern.

For this we can carry out HPLC technique along with mass spectroscopy. At first, the recombinant HBeAg will have to be digested with an enzyme which is active at basic pH so that the enzyme does not prevent the disulfide bond formation. Once the digestion is carried out, the fragments will have to be separated on a C18 column. Once the purified fragments are obtained, they can be subjected to mass spectroscopy so that we can identify specifically which cysteines are disulfide bonded to which ones.

References

1. Zuckerman AJ (1996). [Hepatitis Viruses. In: Baron's Medical Microbiology \(Baron S et al. eds.\)](#) (4th ed.). Univ of Texas Medical Branch. [ISBN 0-9631172-1-1](#).
2. Hepatitis B virus X protein upregulates HSP90alpha expression via activation of c-Myc in human hepatocarcinoma cell line, HepG2
3. Birnbaum, F., and Nassal, M. (1990) J. Virol. 64, 3319-3330
4. Gallina, A., Bonelli, F., Zentilin, L., Rindi, G., Muttini, M., and Milanesi, G. (1989) J. Virol. 63, pp 4645-4652
5. Jian Zheng, Florian Schodel, and Darrell Peterson, THE JOURNAL BIOLOGICAL CHEMISTRY, Vol. 267, No. 13, Issue of May 5, pp. 9422-9429, 1992
6. Florian Schodel, Darrell Peterson, Jian Zhen, Joyce E. Jones, Janice L. Hughes, THE JOURNAL BIOLOGICAL CHEMISTRY, Vol. 268, No. 2, Issue of January 15, pp. 1332-1337, 1993
7. GEORG WASENAUER, JOSEF KOCK, AND HANS-JURGEN SCHLICHT, J. Virol. Vol 67, No. 3, March 1993, pp 1315-1321
8. MICHAEL NASSAL AND ANDREA RIEGER, J. Virol. Vol. 67, No.7, July 1993, pp 4307-4315
9. World Health Organization (WHO) - Hepatitis B Virus - <http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index2.html>

10. Robert H. Purcell , Proc. Natl. Acad. Sci. USA, March 1994, Vol.91, pp 2401-2406
11. ABC of diseases of liver, pancreas, and biliary system - BMJ. 2001 January 20; 322 (7279): 151-153
12. MERCK Manuals - Chronic Hepatitis
13. Mahtab MA, Rahman S, Khan M, Karim F (October 2008). ["Hepatitis B virus genotypes: an overview"](#). *Hbpd Int* 7 (5): 457–64
14. Mahoney, Update on Diagnosis, Management and Prevention of Hepatitis B Virus Infection, Clinical Microbiology Reviews, 1999; CDC
15. Dane, D. S., Cameron, C. H., and Briggs, M. (1970) Lancet 1, 695-698
16. Ou, J.-H., Laub, O., and Rutter, W. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1578 –1582
17. Garcia, P. D., Ou, J.-H., Rutter, W. J., and Walter, P. (1988) J. Cell Biol. 106, 1093–1104
18. Standring, D. N., Ou, J.-H., Masiarz, F. R., and Rutter, W. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8405– 8409
19. Messageot, F., Salhi, S., Eon, P., and Rossignol, J.-M. (2003) J. Biol. Chem. 278, 891–895
20. Patricia MacKay, Janice Lees and Kenneth Murray, Journal of Medical Virology 8: 237 - 243 (1981)

21. Ei Yamada, Hitoshi Ohori and Nakao Ishida, J. gen. Virol. (1981), 55, 75 - 86
22. David Katz, Joseph L. Melnick, F. Blaine Hollinger, J. med. Virol. (1980), Vol. 5, Issue 1, 87 - 100.
23. H. Yoshizawa, Y. Itoh, J. P. Simonetti, T. Takahashi, A. Machida, Y. Miyakawa and M. Mayumi, J. gen. Virol. (1979), 42, 513 - 519.
24. Antibodies: A Laboratory Manual, Ed Harlow and David Lane, 1988
25. Magnus LO, Espmark JA. J. Immunol 1972; 109: 1017 - 1021
26. Takahashi K, Akahane Y, Gotanda T et al. J Immunol 1979; 122: 275 - 279
27. J. Salfeld, E Pfaff, M. Noah and H. Schaller, Journal of Virology, Feb. 1989, p. 798 - 808

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Objective: I belong to a family of entrepreneurs and I wish to be one. The knowledge I have acquired during my Master's program at VCU, in Molecular Biology and Genetics will empower me to setup a Biotech Healthcare Industry or Contract Research Centre in the near future.

Educational Qualification: B. Pharmacy, Al-Ameen College of Pharmacy, Rajiv Gandhi University of Health Sciences, Bangalore. April 2006
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Professional Experience:

Trainee Medical Sales Representative: Undertook field sales training at Troikaa Pharmaceuticals Ltd. as a Medical Sales Representative during the vacation after completion of my schooling in 2001, for a period of three months.

Trainee Researcher: Under took training at the Pharmaceutical Education and Research Development (PERD) Centre, Cellular and Molecular Biology Department in 2006, after completing B.Pharm.

Learnt the Basic Techniques of Molecular Biology.

Worked in the Pharmacogenomics and Proteomics Department. The areas understood by me during my training at PERD Centre are as follows:

DNA Separation by Gel Electrophoresis

Blotting method

Southern Blot Hybridization

Northern Blot Hybridization

DNA Cloning

Genetic Drug Response Profile

Effect of drugs on Gene Expression

Cytochrome 2D6, Cytochrome 2C9, Cytochrome 2C19

Alleles

RNA Splicing
Polymerase Chain Reaction
Biotransformation
Purification of Proteins
Affinity Chromatography
Gel Filtration Chromatography
Ion Exchange Chromatography
Separation of Proteins and Polyacrylamide Gels

Research Analyst: At Troikaa Pharmaceuticals Ltd.

I have acquired proficiency in searching 'prior art', which helps in charting out the course of research activities at Troikaa Pharmaceuticals Ltd. I have also done research work with the research team of Troikaa Pharmaceuticals Ltd. I am one of the co-authors of the inventions titled "Novel Trans-Mucosal Pharmaceutical Compositions and Process For Preparing The Same" (Indian Patent application number- 1704/MUM/2006) and another titled "A Novel Muco-Adhesive Intra-Oral Drug Delivery System and Process for Preparing the same" (Indian Patent application number- 1703/MUM/2006).

While working at Troikaa Pharmaceuticals Ltd., I was on the look out for further training opportunities in the Biotech field. I got selected for training programmes at the Reliance Life Sciences Pvt. Ltd. (www.relbio.com), a state of the art institute working in various areas of biotechnology. Under the conditions of my appointment at Troikaa Pharmaceuticals Ltd., I was granted leave without pay to undergo these trainings.

I underwent trainings at Reliance Life Sciences Pvt. Ltd in Refolding and Purification of Therapeutic Proteins and Molecular Biology Group for a period of 45 days (11th November, 2006 to 26th December, 2006) and 35 days (10th February, 2007 to 15th March, 2007). I learnt techniques like SDS PAGE Gels, Western Blot Technique, Ion Exchange Chromatography, Gel Filtration Chromatography and Hydrophobic Interaction Chromatography. I worked on instruments like AktaPrime plus and Akta Explorer, which are used for the purification process of Therapeutic Proteins.

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