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4.1 HBcAg as a carrier for EDIII-2

HBcAg is known to assemble into virus-like-particles (VLPs), and hence, has been used as a carrier for the display of a number of epitopes on its surface. It is one of the first reported VLP candidates. Selection of HBcAg as a carrier for VLP production becomes highly favorable because of its ability to be synthesized in a prokaryotic host (Pumpens and Grens, 2001). However, to ensure proper folding of the recombinant protein, a eukaryotic expression system, which allows post-translational modifications, is preferred. Eukaryotic systems, like the insect and mammalian cell lines, can be very expensive as expression hosts, and thus, are not economically feasible options. Therefore, methylotrophic yeast, P. pastoris, has become the eukaryotic organism of choice, to enable cost-effective and large-scale production of heterologous proteins (Demain and Vaishnav, 2009). However, P. pastoris as a host, for the production of HBcAg-based chimeric VLP, has not been explored adequately.

The major objective, of the work presented here, has been to evaluate HBcAg as a carrier for exposure of EDIII-2 domain (DENV-2 envelope domain III) on the surface of chimeric HBcAg VLP. In this study, EDIII-2 protein was expressed in P. pastoris in association with the HBcAg as a fusion partner. The rationale for the choice of HBcAg, as a VLP carrier protein, has been presented in Chapter 1 (Sections 1.4.2 and 1.4.3). In the present study, truncated HBcAg protein (aa residues 1-166) was used, and five residues (aa 77-81 of HBcAg), within its Most Immunodominant Region (MIR) or c/el loop, were replaced with 104 aa of EDIII-2. MIR was selected as the most suitable site for the insertion of EDIII-2 because it is the most exposed and immunogenic region of HBcAg. It has been used for the insertion of a number of foreign epitopes, without disturbing VLP formation, as has been discussed in Chapter 1 (Section 1.4.3). Both the proteins, HBcAg and chimeric HBcAg-EDIII-2, were expressed in P. pastoris.

Transformed P. pastoris clones were selected on Zeocin plates and screened by methanol-based induction of the heterologous gene, under carefully optimized conditions. Recombinant proteins were purified to near homogeneity, using Immobilized Metal Affinity Chromatography (IMAC) and characterized. The purified proteins were evaluated for VLP formation by electron microscopy. In order to study the
immunogenicity of these recombinant proteins, they were injected into experimental animals, and the generated immune response was analyzed by ELISA, immunofluorescence, and virus neutralization assays. An outline of the overall experimental strategy adopted is shown in Fig. 4-1.

Fig. 4-1: Outline of the overall experimental strategy adopted in this study involving cloning, expression and purification of recombinant proteins, HBeAg and chimeric HBeAg-EDIII-2, from P. pastoris, and their functional and immunological characterization.
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4.2 Expression and purification of HBcAg protein from P. pastoris

4.2.1 Generation of recombinant plasmid

HBcAg gene (0.565kb), codon-optimized for expression in P. pastoris, was made available, in pMA-T vector, by Geneart. The gene (Fig. 4-2) was designed to encode a protein comprising N-terminal 166 amino acids of HBcAg and a 6x-His tag at its C-terminal end to assist protein purification by Immobilized metal affinity based chromatography.

![Fig. 4-2: Design of HBcAg. Blue and white coloured blocks represent 166 amino acids of HBcAg and a run of six histidine residues, respectively. The numbers in black indicate the aa residue numbers of the protein encoded by the gene. At the N- and C-terminal end of this gene, EcoRI and NotI restriction sites were incorporated, respectively, to enable insertion of this gene in P. pastoris expression vector, pPICZa, between EcoRI and NotI.](image)

**EcoRI**

| GGTACCATGGAGCTTGAATTCATAATGTGACATCGACC | CATATCAAGAAAGATTTGGTGCTTCTATGGAGTTGTTGTCCTTCTTGCCATTCCCAGAGCTTCTACCCATCCGTTAGAGACTTTGGTAACCTCCAGAGCACTGTACTCCACACCACCTGCTTTGAGACAGGCTATTTTGTGGTTGGGGTGAGTTGATGACTTTGGCTACTTGGGTTGGTGGTAACTTGGAGGACCCAATCTCTAGAGACTTGGTTTTCCTACGTTAACACTAACATGGGTTTGAAGTTCAGACAGTTGTTGTGGTTCCACGTTTCCTGTTTGACTTTCGGTAGAGAGACTGGTTGTTGAGTACTTGGTTTCCTGGTGTTTGGATCAGAACTCCACAGGCTTACAGACCACCACGCTCCAATCTTGCCACTTTGCCAGAGACATGTGTTGTTAGAAGAAGAGGTAGATCCCCAGTTAACCCAGTTCCAACTACTGTTTCTCATCACCATAACCACCATTAGTAAAGCGCGCCGCCCTCGAGCTC

**NotI**

![Fig. 4-3: Complete nucleotide sequence of the HBcAg gene. The underlined sequences represent the restriction sites; 'ATG' in bold represents the start codon.](image)

**MSDIDPHYKEFGASMEMLLSFNLPSDFYPSVRDLLDTASALYREALSPEHCTPHHTLREQAILCWGEILMTLAWVGNLEDPIRSDLVVSYTVNNGMLKFROQLWFLFVSCLTGFRETYELVLSFGVWIRTTPQAYRPNNAPIILSTLPETCVVRRRGRSPVNPVPTTSHHHHH**

![Fig. 4-4: Complete amino acid sequence of the encoded HBcAg protein. Sequence in blue represents the 166aa of HBcAg. Sequence in black represents a string of six histidine residues at the C-terminal end of the protein sequence.](image)
The complete nucleotide sequence of this gene is depicted in Fig. 4-3. HBcAg gene was expected to encode 172 aa long protein, corresponding to a molecular weight of ~19.5 kDa. The complete amino acid sequence of the encoded HBcAg protein is represented in Fig. 4-4.

Fig. 4-5: Recombinant plasmid pPICZa-HBcAg carrying HBcAg gene in P. pastoris expression vector, pPICZa, between EcoRI and NotI. In this figure, symbols G, TT, Z, O and P denote HBcAg gene, Transcription Terminator, Zeocin-resistant gene, pUC origin and AOX1 promoter, respectively.

Fig. 4-6: (A) pPICZa-HBcAg was propagated and isolated from DH5α (lane 2), (B) plasmid was confirmed for correct incorporation of HBcAg gene in pPICZa by double digestion with EcoRI and NotI (lane 4). *V and *G represent EcoRI and NotI digested pPICZa vector backbone (3.3kb) and HBcAg gene (0.565kb), respectively. 1kb DNA ladder was run in lanes 1 and 3; their sizes in kb are indicated on the left side of the panel.
In order to express HBcAg in *P. pastoris*, HBcAg gene was cloned in *P. pastoris* expression plasmid, *pPICZa*, between *EcoRI* and *NotI* (Fig. 4-5). The recombinant plasmid *pPICZa-HBcAg* was transformed in DH5α for its propagation. Plasmid was isolated and confirmed by restriction digestion with *EcoRI* and *NotI* (Fig. 4-6). Digestion of the recombinant plasmid with *EcoRI* and *NotI* led to generation of 3.3kb and 0.565kb vector backbone and *HBcAg* gene fragments, respectively, which was in compliance with the predicted pattern (Fig. 4-6, B).

### 4.2.2 Intracellular expression of HBcAg in *P. pastoris*

The *HBcAg* gene was incorporated into the genome of *P. pastoris* in order to achieve its expression in vivo. *HBcAg* gene was cloned in *pPICZa*, between *EcoRI* and *NotI*, linearized with *Sacl* and electroporated into Mut<sup>S</sup> *P. pastoris* host strain, KM71H. The transformation strategy adopted, enabled integration of the *HBcAg*-expression cassette along with Zeocin-resistant gene into the *AOX1* locus of the *P. pastoris* genome. Transformants were selected on 100μg/ml and 500μg/ml Zeocin-containing YPDS plates. Colonies were developed on both, 100μg/ml and 500μg/ml, Zeocin-containing YPDS plates, after incubation at 30°C for 3 days.

![Fig. 4-7: Western Blot for screening of various KM71H transformants expressing 6X-His tagged HBcAg. The blot was developed with anti-His mAb. Lanes 2-5 denote different KM71H clones transformed with HBcAg and selected on 500μg/ml Zeocin; lane 6 denotes uninduced form of the same clone as in lane 5 and lane 7 denotes KM71H clone transformed with *pPICZa* and selected on 500μg/ml Zeocin. Pre-stained protein markers were run in lane 1; their sizes in kDa are denoted on the left side of the panel.](image-url)
Colonies obtained on 500μg/ml Zeocin were screened directly for the expression of HBcAg by methanol-based induction. Test-tube cultures of *P. pastoris* transformants were grown overnight and induced with 2% methanol to check for expression of the recombinant HBcAg protein by Western blot, developed with anti-His mAb (Fig. 4-7). Based on this result, the clone in lane 4 was selected for further studies.

### 4.2.3 Purification of recombinant HBcAg

The His-tagged HBcAg protein was purified by Ni-NTA chromatography. The purification strategy involved lysis of induced biomass in Guanidine-HCl containing lysis buffer, followed by denaturing Ni-NTA chromatography. An aliquot of the alternate peak fractions from this chromatography was analyzed on a denaturing polyacrylamide gel and visualized by coomassie stain (Fig. 4-8). The purified fractions were pooled and dialysed, against 25mM Sodium bicarbonate buffer, pH 9.2, by step-wise removal of urea from 8M to 0M. The performance of this purification strategy, adopted for HBcAg, is summarized in Table 4-1.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Specific protein (mg)</th>
<th>Purity (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell extract</td>
<td>2500</td>
<td>110</td>
<td>4.4</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA chromatography</td>
<td>45</td>
<td>41</td>
<td>~91</td>
<td>37.3</td>
</tr>
</tbody>
</table>

* Total protein was calculated by BCA method using bovine serum albumin as reference  
† Calculated by densitometric analysis  
‡ Purity was taken as the percentage ratio between specific protein and total protein  
§ Recovery was calculated as the ratio of specific protein content post-purification to specific protein content in the whole cell extract

The data presented in Fig. 4-7 demonstrates that *P. pastoris* was able to express His-tagged HBcAg protein, which could be further purified by Ni-NTA chromatography (Fig. 4-8). Further, the data, presented in Table 4-1 and SDS-PAGE analysis (Fig. 4-8 inset), shows that the purification strategy, adopted for HBcAg protein, resulted in the production of 41mg of purified protein from 1L of BMGY culture, with a recovery of 37%. The purity of the final preparation was judged to be ~ 91%.
4.2.4 Conclusions

- The gene encoding N-terminal 166aa of HBcAg, with a 6x-His tag at its C-terminal end, was artificially synthesized by Geneart.
- The gene was integrated into AOX1 locus of the genome of the Mut$^S$ strain, KM71H, of P. pastoris.
- Recombinant HBcAg protein was expressed in P. pastoris and purified, to near homogeneity, by denaturing Ni-NTA chromatography.
4.3 Expression and purification of HBcAg-EDIII-2 protein from *P. pastoris*

4.3.1 Generation of recombinant plasmid

*HBcAg-EDIII-2* gene (0.812kb), codon-optimized for expression in *P. pastoris*, was made available, in *pMA-T* vector, by Geneart. Fig. 4-9 gives a schematic representation of the gene design, in which EDIII-2 domain was inserted at the MIR region of HBcAg to optimally expose it on the surface of HBcAg VLP.

![Fig. 4-9: Design of HBcAg-EDIII-2 gene. Blue, pink and white blocks represent HBcAg, EDIII-2 and a stretch of seven histidine residues, respectively. The numbers in black indicate the aa residue number of the protein encoded by this gene. At the N- and C-terminal end of this gene, EcoRI and NotI restriction sites were incorporated, respectively, to enable insertion of this gene in P. pastoris expression vector, pPICZa, between EcoRI and NotI.](image)

Fig. 4-10 gives the complete nucleotide sequence of the gene encoding HBcAg-EDIII-2, in which a stretch of 104aa of EDIII-2, was incorporated between 76\(^{th}\) and 82\(^{nd}\) aa of HBcAg, thus replacing five residues (77-81aa) from HBcAg. A run of seven histidine residues was also incorporated at its C-terminal end to enable its purification by Immobilized Metal Affinity Chromatography. *HBcAg-EDIII-2* gene was expected to encode 272aa long protein, corresponding to a molecular weight of ~31kDa. The complete amino acid sequence of the encoded HBcAg-EDIII-2 protein is represented in Fig. 4-11.

In order to express this gene in *P. pastoris*, *HBcAg-EDIII-2* gene was cloned in *P. pastoris* expression plasmid, *pPICZa*, between EcoRI and NotI and transformed into DH5\(\alpha\) for its propagation. Fig. 4-12 gives a schematic representation of the recombinant plasmid *pPICZa-HBcAg-EDIII-2*. The recombinant plasmid was isolated from DH5\(\alpha\) and confirmed by double digestion with EcoRI and NotI (Fig. 4-13). EcoRI-NotI digestion of the *pPICZa-HBcAg-EDIII-2* plasmid generated 3.3kb and 0.812kb fragments of vector backbone and *HBcAg-EDIII-2* gene, respectively, which was in compliance with the predicted pattern (Fig. 4-13, B).
**Results**

**EcoRI**

<table>
<thead>
<tr>
<th>EcoRI</th>
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| GGGCGAATTGGGTACCGAATTCTGCTGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGACATCGA**

*Fig. 4-10: Complete nucleotide sequence of HBcAg-EDIII-2 gene. The underlined sequences represent the restriction sites; 'ATG' in bold represents the start codon.*

| MSDIDPYKFEGASMELLFLPSDFYPSPVRDSLTDASALREALESPHECTPHHTALRQAIIWCWE |
| LMTLAATWGGHMSMTCCGCXKVKEIAETQHGTIVSRVYEGSICPSHTFIMDLEKRVHVLGR |
| LTTVNPVEKDSVPVIEAEPFEDSYIILLVEPFGKILNWFKKGSSIQGRDLSWYVNTMGL |
| KERQILWIFIVSTTFGRETIVELYVFSGFWIRTPQARYRPPANNLSTLPETCVRRRGRSVPNV |
| PTIVSHHHHHHHH |

*Fig. 4-11: Complete amino acid sequence of the encoded HBcAg-EDIII-2 protein. Sequences in blue and pink represent HBcAg and EDIII-2 amino acid sequence, respectively. Sequence in black represents the 7X-His tag at the C-terminal end of the protein sequence.*

**Fig. 4-12: Recombinant plasmid, pPICZa-HBcAg-EDIII-2, with HBcAg-EDIII-2 gene inserted between EcoRI and NotI. In this figure, symbols G, TT, Z, O and P denote HBcAg-EDIII-2 gene, Transcription Terminator, Zeocin-resistant gene, pUC origin and AOX1 promoter, respectively.**

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Fig. 4-13: (A) pPICZa-HBcAg-EDIII-2 was propagated and isolated from DH5α (lane 2), (B) plasmid was confirmed for correct incorporation of HBcAg-EDIII-2 gene in pPICZa by double digestion with EcoRI and NotI (lane 4). *V and *G represent pPICZa vector backbone (3.3kb) and HBcAg-EDIII-2 gene fragment (0.812kb), after double digestion of pPICZa-HBcAg-EDIII-2 with EcoRI and NotI. 1kb DNA ladder was run in lanes 1 and 3; their sizes in kb are indicated on the left side of the panel.

4.3.2 Intracellular expression of HBcAg-EDIII-2 protein in P. pastoris

For in vivo expression of HBcAg-EDIII-2 in P. pastoris, the recombinant plasmid pPICZa-HBcAg-EDIII-2 was linearized with SacI and electroporated into MutS strain of P. pastoris, KM71H. The transformation strategy adopted, allowed the integration of chimeric protein-expression cassette along with the Zeocin-resistant gene into the AOX1 locus of the P. pastoris genome. Transformants were selected on 100µg/ml and 500µg/ml Zeocin-containing YPDS plates. After incubation at 30°C for 3 days, few colonies developed on 100µg/ml Zeocin plate, while only one colony developed on 500µg/ml Zeocin plate. Three colonies from 100µg/ml Zeocin plate, and one from 500µg/ml Zeocin plate, were screened directly for the expression of HBcAg-EDIII-2, by methanol-based induction. Test-tube cultures of P. pastoris transformants were grown overnight and induced with 2% methanol to check for expression of the recombinant fusion protein by Western blot, developed with an in-house anti-EDIII mAb (Fig. 4-14). Based on this result, clone in lane 3, selected on 100µg/ml Zeocin, was chosen for further study.
Fig. 4-14: Western Blot for screening of various KM71H transformants expressing His-tagged HBcAg-EDIII-2. The blot was developed with an in-house anti-EDIII mAb. Lanes 2-5 denote different KM71H clones transformed with HBcAg-EDIII-2; lane 6 denotes uninduced form of the same clone as in lane 5. Clones in lane 2-4 were selected on 100μg/ml Zeocin, while clone in lane 5 was selected on 500μg/ml Zeocin. Pre-stained protein markers were run in lane 1; their sizes in kDa are denoted on the left side of the panel.

4.3.3 Optimization of HBcAg-EDIII-2 expression in shake-flask cultures

With the aim to achieve optimum expression of HBcAg-EDIII-2 in *P. pastoris*, concentration of methanol and duration of induction were optimized. The expression of HBcAg-EDIII-2 was measured at varying concentrations of methanol, ranging from 0.5-2%, with the duration of induction kept constant at 72hr (Fig. 4-15, lanes 2-5).

At 0.5% (v/v) methanol, the level of protein expression was low. A significant increase in the expression level was observed with increase in methanol concentration to 1% (v/v). Further increase in methanol concentration to 1.5 and 2% resulted in modest increase in the level of expression of HBcAg-EDIII-2.

The expression of recombinant protein was also monitored as a function of duration of induction, with the methanol feed of 2% kept constant (Fig. 4-15, lanes 6-9). In this experiment, a logarithmically growing *P. pastoris* culture was induced with 2% (v/v) methanol. Aliquots of the culture were withdrawn at 24hr intervals till 72hr and analyzed by Western Blot developed with anti-EDIII mAb. Fig. 4-15 indicates that the expression level of HBcAg-EDIII-2 increased with increase in duration of induction from 0hr to 72hr.
Results

Methanol concentrations beyond 2% and induction time longer than 72hr were not tested, as a reduction in recombinant protein production under these conditions has been reported, which may be associated with methanol toxicity and manifestation of proteolytic activity, respectively (Batra et al., 2010). Thus, under the current experimental set-up, methanol concentrations of 2% (v/v) and induction for 72hr resulted in maximum expression of this protein. Hence, induction was carried out in the presence of 2% (v/v) methanol for 72hr for subsequent experiments.

4.3.4 Localization of HBcAg-EDIII-2

The induced recombinant *P. pastoris* cells, expressing HBcAg-EDIII-2 protein, were lysed overnight in native lysis buffer, with glass beads on a thermomixer. The supernatant was separated and stored at -20°C, while the protein present in the pellet was extracted with extraction buffer, containing 6M Guanidine-HCl, for 4hr. HBcAg-EDIII-2 protein, in soluble (native) and insoluble (membrane) extracts, was visualized through western blot and estimated through Ni-NTA His-sorb ELISA. It was found that the protein of interest was present, only in the insoluble membrane extract (Fig. 4-16).
Results

A

B

Fig. 4-16: Presence of HBCAg-EDIII-2 protein in soluble and membrane extract was detected through (A) Western Blot and (B) Ni-NTA His-sorb ELISA, developed with in-house anti-EDIII mAb. (A) Soluble fraction and insoluble membrane extract were run in lanes 2 and 3, respectively. Pre-stained markers were run in lane 1; their sizes in kDa are denoted on the left side of the panel. (B) Red and blue bars represent HBCAg-EDIII-2 protein present in soluble and membrane extracts, respectively.

4.3.5 Purification of recombinant HBCAg-EDIII-2

Since HBCAg-EDIII-2 protein was found to be absent in soluble fraction (Fig. 4-16), the protein was purified from the membranous extract of the induced cells by denaturing Ni-NTA chromatography (Fig. 4-17). An aliquot of the alternate peak fractions from this chromatography was analyzed on a denaturing polyacrylamide gel and visualized by coomassie stain (Fig. 4-17, inset). Purified protein-containing fractions were pooled and dialysed against 25mM Sodium bicarbonate buffer, pH 9.2, by step-wise removal of urea from 8M to 0M. The purity and recovery of protein has been summarized in Table 4-2.

The data presented in Fig. 4-14 demonstrates that P. pastoris was able to express the intended chimeric protein, HBCAg-EDIII-2. Induced biomass of the selected clone was prepared under optimized conditions of 2% methanol for 72hr (Fig. 4-15). The cells were lysed and proteins were extracted from membrane (Fig. 4-16). The extract was subjected to denaturing Ni-NTA chromatography for purification (Fig. 4-17). Further, the data, presented in Table 4-2 and SDS-PAGE analysis (Fig. 4-17, inset), shows that the purification strategy, adopted for HBCAg-EDIII-2 chimeric protein, could efficiently
recover 37% of the protein with a purity of ~93%. 26mg of purified protein was obtained from 1L of BMGY culture.

![Graph showing chromatographic elution profile and SDS-PAGE analysis of the recombinant HBcAg-EDIII-2 protein.](image)

*Fig. 4-17: Immobilized metal affinity (Ni-NTA) chromatographic elution profile and SDS-PAGE analysis of the recombinant HBcAg-EDIII-2 protein. An aliquot of the alternate peak fractions was run in lanes 3-8; an aliquot of wash with buffer of pH 5.9 was run in lane 2; low molecular weight protein markers were run in lane 1; their sizes in kDa are denoted on the left side of the panel.*

Table 4-2: Purification summary of recombinant HBcAg-EDIII-2 chimeric protein from 1L of BMGY culture

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Specific protein (mg)</th>
<th>Purity (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native extract</td>
<td>1500</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Membrane extract</td>
<td>733</td>
<td>70</td>
<td>9.55</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA chromatography</td>
<td>28</td>
<td>26</td>
<td>92.86</td>
<td>37</td>
</tr>
</tbody>
</table>

* Total protein was calculated by BCA method using bovine serum albumin as reference
† Calculated by Ni-NTA His-sorb ELISA using a previously characterized protein as reference
‡ Purity was taken as the percentage ratio between specific protein and total protein
¶ Recovery was calculated as the ratio of specific protein content post-purification to specific protein content in the membrane extract
4.3.6 Conclusions

- The 104aa long envelope domain III of DENV-2 (EDIII-2) was incorporated at the MIR of C-terminally truncated HBcAg between its 76th and 82nd residue. A 7x-His tag was also incorporated at the C-terminal end of the protein to assist its purification by Immobilized Metal Affinity Chromatography. The designed gene was artificially synthesized by Geneart.

- The chimeric gene, HBcAg-EDIII-2, was integrated into the AOXI locus of the genome of Mut® P. pastoris, KM71H.

- Recombinant P. pastoris was able to express HBcAg-EDIII-2 protein.

- Recombinant HBcAg-EDIII-2 protein expression was maximal when P. pastoris clone was induced with 2% (v/v) methanol for 72hr.

- HBcAg-EDIII-2 chimeric protein was found to be present in the insoluble membrane extract.

- The HBcAg-EDIII-2 protein expressed in P. pastoris was purified, to near homogeneity, from the membrane extract, by denaturing Ni-NTA chromatography.
4.4 Characterization of recombinant proteins

4.4.1 Size exclusion chromatography to detect the presence of higher order structures

The behavior of purified HBcAg-EDIII-2 protein in size exclusion chromatography was examined with the aim to determine the extent of presence of higher order structures, as compared to its monomeric form. Fig. 4-18 depicts size exclusion chromatographic elution profile, which depicts a major peak in the void volume, and a minor shoulder peak outside the void volume. Elution of HBcAg-EDIII-2 in the void volume indicates the presence of higher order structures. The shoulder peak indicates that the protein in minor peak is probably morphologically different from the protein in the major peak.

![Size exclusion chromatographic elution profile and SDS-PAGE analysis of the peak fractions as inset. Lane 2 depicts the major peak fraction, lanes 3-5 denote alternate fractions from the minor peak, lanes 6-8 denote alternate fractions following the minor peak. The horizontal bar indicates the void volume.](image-url)
4.4.2 Electron-microscopic evaluation of purified proteins for the formation of VLPs

Purified proteins, HBCAg and HBCAg-EDIII-2, were evaluated for the formation of VLPs by electron microscopy. Purified proteins were coated on grids and negatively stained, using uranyl acetate. HBCAg was observed to form VLPs of ~30nm size (Fig. 4-19, A), while HBCAg-EDIII-2 chimeric protein assembled to form ~40nm sized VLPs (Fig. 4-19, B).

![Fig. 4-19: Electron-microscopic evaluation of purified proteins, HBCAg (A), and HBCAg-EDIII-2 (B). VLPs of ~30 and 40nm were observed for HBCAg and HBCAg-EDIII-2, respectively.]

4.4.3 Immunological evaluation of purified proteins

Groups of Balb/c mice were, separately, immunized with purified HBCAg, HBCAg-EDIII-2 and EDIII-2 proteins, bound on alum (alhydrogel) adjuvant, in order to study the antibody response generated against them. A control group of mice was injected with PBS and alum mix. Immune response generated by these antigens was analyzed by ELISA, using post-second boost immunization sera (Fig. 4-20, A-C). Three sets of ELISA were performed, where HBCAg (green curve), HBCAg-EDIII-2 (purple curve), EDIII-2 (red curve) and PBS (blue curve) antisera were analyzed on (A) HBCAg, (B) EDIII-2 and (C) HBCAg-EDIII-2 antigens (Fig. 4-20, A-C). It is evident from the data that HBCAg-EDIII-2 is immunogenic, since it was able to elicit high titer antibodies. Both, HBCAg and HBCAg-EDIII-2 antisera, could recognize HBCAg antigen (Fig. 4-20, A). The two sera could also recognize HBCAg-EDIII-2 antigen (Fig. 4-20, C). The presence of anti-EDIII-2 antibodies in HBCAg-EDIII-2 antisera was confirmed by
Results

analyzing the sera on, in-house purified, EDIII-2 protein (Batra et al., 2010), which revealed that HBcAg-EDIII-2 antisera contained EDIII-2-specific antibodies (Fig. 4-20, B). It also indicates that EDIII-2 moiety in HBcAg-EDIII-2 VLPs is exposed enough to elicit EDIII-2 specific antibodies. Both, mock (PBS-immunized) and HBcAg antisera, could not recognize EDIII-2 antigen. Mock antiserum was non-reactive in all the three ELISAs. It can be observed that EDIII-2 antiserum elicited higher titers than HBcAg-EDIII-2 antiserum against both, EDIII-2 (Fig. 4-20, B) and HBcAg-EDIII-2 (Fig. 4-20, C).

The antibody titer of post-first and second boost HBcAg-EDIII-2 antisera was evaluated against HBcAg-EDIII-2 (Fig. 4-20, D). It was observed that post-second boost sera (purple curve) had significantly higher antibody titer than post-first boost sera (orange
curve). Mock antisera (post-first and second boost) did not show any reactivity (Fig. 4-20, D).

4.4.4 Competitive ELISA to examine the exposure of Dengue Envelope domain III on the surface of HBcAg-EDIII-2 VLPs

It is well documented that antigens inserted into the c/e1 loop, or the MIR, of HBcAg, tend to be displayed on the surface of the chimeric VLPs (Pumpens and Grens, 2001). The assessment, whether this is true for HBcAg-EDIII-2 chimeric VLP as well, was made through a competitive ELISA assay. In this assay, the residual antigen-binding activity of different anti-EDIII antibodies, pre-incubated with HBcAg-EDIII-2 antigen, was measured using monomeric EDIII-2 as the coating antigen. The rationale was that if the EDIII-2 moiety of the chimeric VLP was accessible to anti-EDIII antibody, it would render the antigen-binding sites on the latter unavailable for binding to the coated EDIII-2 antigen in the microtiter wells. Thus, a reduction in ELISA reactivity in this assay would indicate exposure and accessibility of EDIII-2 moiety (of the fusion antigen) on the surface of chimeric VLP, reducing the availability of anti-EDIII antibody to bind to the coated EDIII-2 antigen. This notion is borne out by the data presented in Fig. 4-21.

![Fig. 4-21: Competitive ELISA. Varying concentrations of purified HBcAg (red squares) and HBcAg-EDIII-2 (blue circles) VLPs were pre-incubated with (A) HBcAg-EDIII-2 antiserum, (B) anti-EDIII mAb and (C) EDIII-T antiserum. Residual antibodies in the pre-incubation mix were captured on ELISA plates coated with purified P. pastoris-expressed EDIII-2 protein and revealed using anti-mouse IgG-peroxidase in conjunction with TMB (soluble) substrate. ELISA reactivity in the absence of any added protein in the pre-incubation step was taken to represent 100%. The black line represents linear regression curve for each of the data sets. The regression equation and correlation coefficient ($R^2$) have been indicated for HBcAg-EDIII-2 data points.](image-url)
Results

When an in-house EDIII-specific mAb was tested in this assay (Fig. 4-21, B), its reactivity to the EDIII-2 coated antigen was inversely proportional to the concentration of HBcAg-EDIII-2 VLP it was pre-treated with. However, pre-treatment of this mAb with HBcAg VLPs (lacking EDIII-2) did not affect its ELISA reactivity towards the EDIII-2 coated antigen. Similar results were obtained when this experiment was performed with a murine polyclonal serum, raised against (A) HBcAg-EDIII-2 chimeric antigen (Fig. 4-21, A), and (C) chimeric EDIII-T antigen containing the EDIIIs of the four DENV serotypes linked in tandem (Etemad et al., 2008) (Fig. 4-21, C). This suggests that the EDIII-2 moiety on the chimeric VLP is accessible on the surface, and therefore, capable of recognizing and binding to the monoclonal and polyclonal anti-EDIII antibodies.

4.4.5 HBcAg-EDIII-2 elicited antibodies recognize dengue virus

Presence of DENV-2-specific antibodies in the polyclonal sera was evaluated by indirect immunofluorescence assay. It was observed that the HBcAg-EDIII-2 antiserum could recognize DENV-2 (Fig. 4-22 D), suggesting that the antiserum contained antibodies specific to DENV-2. Mock (PBS immunized) and HBcAg antiserum (Fig. 4-22, A and B), as expected, failed to detect the virus. Interestingly, EDIII-2 antiserum failed to recognize the virus efficiently (Fig. 4-22, C), even though it elicited much higher antibody titers (estimated through ELISA) than HBcAg-EDIII-2 antiserum (Fig. 4-20, B and C).

4.4.6 Antibodies elicited by HBcAg-EDIII-2 VLPs neutralize dengue virus

Ability of HBcAg-EDIII-2 antiserum to neutralize DENV-2 was evaluated through Plaque Reduction Neutralization Test (PRNT). HBcAg-EDIII-2 antiserum was found to contain virus-neutralizing antibodies against DENV-2. A PRNT_{50} titer of ~1:70 was obtained. EDIII-2 antiserum, like mock and HBcAg antisera, did not manifest any discernible DENV-2 neutralizing antibody titers (PRNT_{50} titer ~1:25) (Table 4-3). After correcting PRNT_{50} titer of each antiserum with that of mock, the titer of only HBcAg-EDIII-2 antiserum appeared to be significant (~1:40).
Fig. 4-22: Determination of presence of DENV-2 recognizing antibodies in the (A) Mock (PBS)-, (B) HBcAg-, (C) EDIII-2- and (D) HBcAg-EDIII-2-polyclonal sera, by immunofluorescence assay.

Table 4-3: PRNT$_{50}$ titers of the antisera.

<table>
<thead>
<tr>
<th>ANTISERA</th>
<th>PRNT$_{50}$</th>
<th>Mock-corrected PRNT$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>HBcAg</td>
<td>26</td>
<td>-1</td>
</tr>
<tr>
<td>EDIII-2</td>
<td>23</td>
<td>-3</td>
</tr>
<tr>
<td>HBcAg-EDIII-2</td>
<td>70</td>
<td>43</td>
</tr>
</tbody>
</table>

4.4.7 Conclusions

- HBcAg-EDIII-2 protein forms VLPs.
- HBcAg-EDIII-2 is immunogenic and elicits EDIII-2-specific antibodies in Balb/c.
- EDIII-2 moiety of HBcAg-EDIII-2 is exposed on its VLP surface.
- HBcAg-EDIII-2 antisera could recognize DENV-2.
- HBcAg-EDIII-2 elicits neutralizing antibodies against DENV-2.