MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 10, No 4, December 2016, p 119-124

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.10.4.1

# Cloning and Expression of Small Hepatitis B Surface Antigen (sHBsAg) In *Hansenula polymorpha*

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Recombinant small hepatitis B surface antigen (sHBsAg) is used as a vaccine component to prevent hepatitis B virus infection. As an attempt to produce local recombinant sHBsAg, a PCR-amplified DNA fragment encoding Indonesia sHBsAg which belongs to B genotype and adw2 subtype was cloned into *Hansenula polymorpha* expression vector pHIPX4 by using recombination method. The resulted pHIPX4-sHBsAg was integrated into the alcohol oxidase locus of *H. polymorpha* NCYC495 genome and the sHBsAg expression was regulated under the control of *H. polymorpha AOX* promoter. *H. polymorpha* NCYC495 carrying the sHBsAg coding sequence was grown in mineral medium and methanol 0.5% (v/v) was added to induce the expression of recombinant sHBsAg. The expression of sHBsAg was detected by HBsAg diagnostic kit test, ELISA, and Western blot analysis.

Key words: *AOX* promoter, *Hansenula polymorpha*, hepatitis B, *sHBsAg*

Antigen permukaan virus hepatitis B berukuran kecil (sHBsAg) rekombinan digunakan sebagai komponen vaksin untuk mencegah infeksi virus hepatitis B. Sebagai upaya untuk memproduksi sHBsAg rekombinan lokal, fragmen DNA amplifikasi PCR yang mengkode sHBsAg Indonesia yang tergolong ke dalam genotipe B dan subtipe adw2 diklon ke dalam vektor ekspresi *Hansenula polymorpha* pHIPX4 dengan menggunakan metode rekombinasi. Plasmid rekombinan pHIPX4-sHBsAg terintegrasi ke dalam lokus alkohol oksidase (*AOX*) dari genom *H. polymorpha* NCYC495 dan ekspresi sHBsAg diregulasi di bawah kendali promotor *AOX H. polymorpha*. *H. polymorpha* NCYC495 yang membawa urutan kode sHBsAg ditumbuhkan di dalam medium mineral dan metanol 0,5% (v/v) ditambahkan untuk menginduksi ekspresi sHBsAg rekombinan. Ekspresi sHBsAg dideteksi melalui uji kit diagnostik HBsAg, ELISA, dan Western blot.

Kata kunci: promotor *AOX*, *Hansenula polymorpha*, hepatitis B, *sHBsAg*

Hepatitis B virus infection can cause a chronic liver disease. It is estimated that 240 million people are chronically infected with hepatitis B and more than 786 000 people die every year due to complication of hepatitis B, including cirrhosis and liver cancer (World Health Organization c2015). In Indonesia, there are about 28 million people infected with hepatitis B and 14 million people are potentially becoming chronic with 10% progress into hepatocellular carcinoma (Kementerian Kesehatan Republik Indonesia 2013).

The most effective approach to control and prevent the spreading of hepatitis B is by vaccination (WHO 2010). In Indonesia, hepatitis B vaccine has been integrated into national immunization programmes since 1997 (Kementerian Kesehatan Republik Indonesia 2013). Therefore, in order to fulfill the need of hepatitis B antigen, here we report the production of local recombinant small hepatitis B surface antigen (sHBsAg) using *Hansenula polymorpha* expression system.

*H. polymorpha* expression system offers more advantages than non-methylotrophic yeast expression system, such as *Saccharomyces cerevisiae*. *H. polymorpha* expression system has a strong and tightly-regulated alcohol oxidase (*AOX*) inducible promoter, and a high-frequency of non-homologous recombination (Kang and Gelissen 2005). Moreover, *H. polymorpha* does not hyperglycosylate protein, is able to grow in simple medium, and has thermotolerant property (Kang and Gelissen 2005; Reinders *et al.* 1999).

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This paper described cloning of sHBsAg coding sequence into *H. polymorpha* expression vector pHIPX4 and expression of the sHBsAg in *H. polymorpha* NCYC495. The sHBsAg expression was analysed with HBsAg diagnostic kit test, ELISA, and Western blot.

## MATERIALS AND METHODS

**Construction of pHIPX4-sHBsAg**. The sHBsAg coding sequence of hepatitis B virus was amplified by PCR method using pPICZα-A-HBsAg (Nurfitriani 2012) as a template and a set of primers SF (CTAAAGTACAAAAACAAGCTTATGGAGAAC ACGCATCAGG) and SR (GATCGATCCTCTAGAG TCGACTTAAATGTATACCCAAAGAC). The amplified sHBsAg DNA fragment and pHIPX4 previously digested with *Hind*III/*Sal*I were homologously recombined according to CloneEZ® PCR Cloning Kit procedure (GenScript, USA). The resulted recombinant plasmid was designated as pHIPX4-sHBsAg. The nucleotide sequence of sHBsAg in pHIPX4-sHBsAg was determined by dideoxy-chain termination method (Macrogen, Korea).

**Transformation of *H. polymorpha* NCYC495 and Recombinant sHBsAg Expression**. *H. polymorpha* NCYC495 *leu*1.1 was transformed with recombinant plasmid pHIPX4-sHBsAg, which had been linearized with *Sca*I, according to the method described by Faber *et al*. (Faber *et al.* 1994). *H. polymorpha* NCYC495 transformants were grown on YND medium, which composed of 0.67% (w/v) yeast nitrogen base without amino acids, 1% (w/v) dextrose, and 1.6% (w/v) bacto agar. PCR colony was performed using primers SF (CTAAAGTACAAAAA CAAGCTTATGGAGAACACGCATCAGG) and SR (GATCGATCC TCTAGAGTCGACTTAAATGTATA CCCAAGAC) to verify plasmid integration into the *H. polymorpha* NCYC495 genome.

The transformants were precultured in 5 mL mineral medium (van Dijken *et al.* 1976) containing 0.25% (w/v) glucose and incubated with shaking at 200 rpm for 18 h at 37 °C. The culture was then centrifuged at 2,900 *g* and the whole pellet cell were inoculated in 50 mL fresh mineral medium containing 0.5% (v/v) methanol as the sole carbon source. The culture was incubated with shaking at 200 rpm, 37 °C. To induce recombinant sHBsAg expression, 0.5% (v/v) methanol was added to the culture medium every 22 h for 66 h.

The yeast cells were harvested by centrifugation at 2900 *g*, 4 °C, and the cell pellet was resuspended in lysis buffer which contained 10 mM potassium phosphate pH 8, 500 mM NaCl, 5 mM EDTA, 8% (v/v) glycerol, 1% (v/v) Triton X-100, and 1% (v/v) leupeptin 2.5 μg $mL^{-1}$. *g*, The cells were lysed by manual grinding in liquid nitrogen. The protein was collected by centrifugation at 7200 *g*, 4 °C for 10 min.

**HBsAg Diagnostic Kit Test**. Recombinant sHBsAg was analysed with HBsAg diagnostic kit test, *Uji Hepatitis BsAg* (Pakar Biomedika Indonesia, Indonesia). The test was conducted using 50 μL protein samples.

**ELISA.** Recombinant sHBsAg was examined with commercial ELISA kit, Murex HBsAg Version 3 (DiaSorin, Italy). ELISA was performed using 1 μg protein samples and 75 μL of ELISA negative and positive control. All protein samples and controls were analysed in duplicate.

The negative and positive controls were provided by the kit manufacturer. The negative control was a normal human serum diluted in a buffer containing protein of bovine origin and 0.05% (w/v) Bronidox® preservative, while the positive control was an inactivated human serum diluted in a buffer containing protein of bovine origin and 0.05% (w/v) Bronidox® preservative. The ELISA threshold value was calculated according to the instruction of ELISA kit manufacturer by adding a value of 0.050 to the $A_{450}$ of negative control provided in the ELISA kit.

**Western Blot**. 10 μg protein samples were first separated on a 12% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred onto nitrocellulose membrane and then blocked with Roti Block (Carl Roth, Germany) for 2 h at room temperature.

The sHBsAg protein was detected using monoclonal anti-HBsAg (Virostat, USA), goat anti-mouse antibody-alkaline phosphatase conjugate (Biorad, USA), and visualized with NBT/BCIP. Purified recombinant sHBsAg, produced in-house by PT. Bio Farma (Persero), was used as a positive control.

## RESULTS

**Integration of Linearized pHIPX4-sHBsAg into *H. polymorpha* NCYC495 Genome**. The sHBsAg coding sequence was subcloned into *H. polymorpha* expression vector pHIPX4 which has *Saccharomyces cerevisiae* LEU2 gene under its endogenous promoter. The resulted recombinant plasmid pHIPX4-sHBsAg

(Fig 1) was verified by restriction enzyme analysis using *Hind*III and *Sal*I (Fig 2A). The digested pHIPX4-sHBsAg gave two DNA fragments with the size of 7.0 kb and 0.7 kb which represented the pHIPX4 vector and sHBsAg insert, respectively. The recombinant plasmid pHIPX4-sHBsAg was linearized in the promoter AOX region to allow its integration in the AOX locus of *H. polymorpha* NCYC495 genome which enabled the transformants to grow on minimal medium without leucine. PCR colony of *H. polymorpha* NCYC495 transformants gave a 0.7 kb DNA fragment which confirmed the presence of sHBsAg coding sequence in the *H. polymorpha* NCYC495 genome (Fig 2B).

**Identification of Recombinant sHBsAg.** Crude protein extracts from two *H. polymorpha* NCYC495 transformants (designated as S1 and S2) were evaluated using commercial HBsAg diagnostic kit. The S1 and S2 crude protein extracts gave positive interaction with HBsAg antibody, whereas the interaction was negative for crude protein extract from *H. polymorpha* NCYC495 (Table 1). This result indicated that crude protein extracts of S1 and S2 contained recombinant sHBsAg.

Further identification of the expression of recombinant sHBsAg in S1 and S2 was conducted using ELISA. The crude protein extracts from S1 and S2 gave the value of $A_{450}$ that was higher than ELISA threshold value (Fig 3A). In contrast, the crude protein extract from *H. polymorpha* NCYC495 had lower $A_{450}$ value than that of the threshold value (Fig 3A). This ELISA result suggested the presence of recombinant sHBsAg in S1 and S2.

Western blot analysis was performed to demonstrate

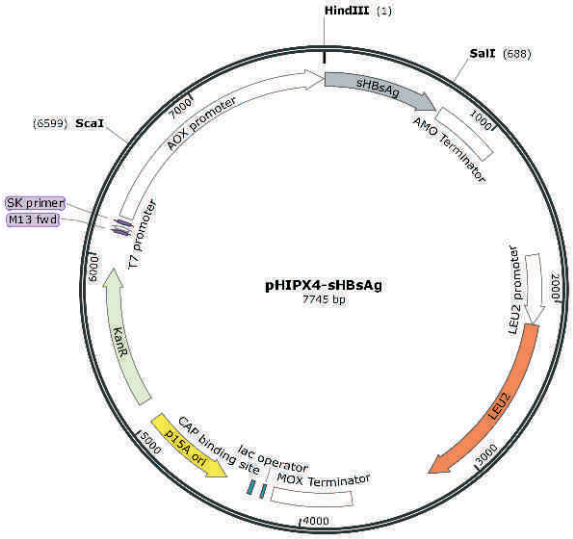


Fig 1 pHIPX4-sHBsAg recombinant plasmid. The plasmid map was generated using SnapGene® Viewer 3.0.1.

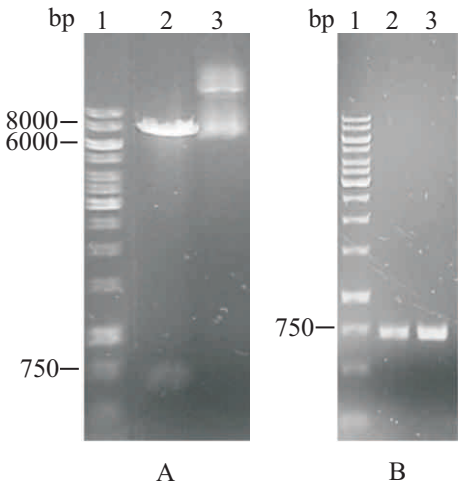


Fig 2 Restriction enzyme analysis of pHIPX4-sHBsAg recombinant plasmid with its uncut control (A) and colony PCR of *H. polymorpha* NCYC495-pHIPX4-sHBsAg (B). (A) Lane 1, DNA ladder; 2, pHIPX4-sHBsAg digested with *Hind*III and *Sal*I; 3, uncut pHIPX4-sHBsAg. (B) Lane 1, DNA ladder; 2-3, positive transformant.

Table 1. HBsAg diagnostic kit test of crude protein extracts

| Sample | NCYC495 | S1 | S2 |
|---|---|---|---|
| Interaction | – | + | + |

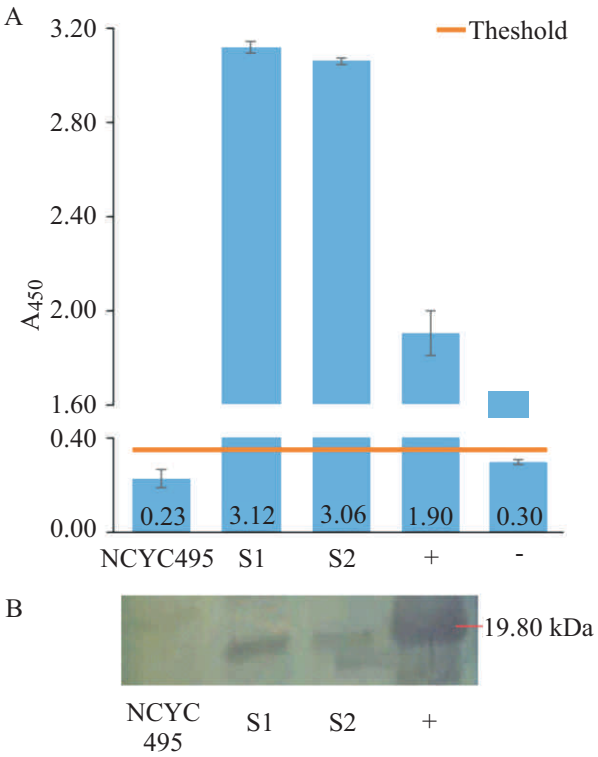


Fig 3 ELISA (A) and Western blot analysis (B) of crude protein extracts from *H. polymorpha* NCYC495-pHIPX4-sHBsAg (S1 and S2) and *H. polymorpha* NCYC495. All ELISA value bar represent an average value of two repeated assay.

the expression of sHBsAg in *H. polymorpha* NCYC495 pHIPX4-sHBsAg. A protein band at molecular weight of 19.8 kDa appeared in the crude protein extracts from S1 and S2, as well as in the purified sHBsAg (Fig 3B). As expected, there was no protein band detected in the *H. polymorpha* NCYC495 crude protein. Taken together, this result confirmed that S1 and S2 produced recombinant sHBsAg.

The negative control of ELISA is a normal human serum, while the positive control is an inactivated human serum. The positive control of Western blot is purified recombinant sHBsAg from PT. Bio Farma (Persero).

## DISCUSSION

The Indonesia sHBsAg sequence used in this work was derived from local clinical isolates of Hasan Sadikin Hospital Bandung in which the virus has B genotype and adw2 subtype (Suhandono *et al.* 2007). Amino acid sequence alignment of the "a" determinant region of several sHBsAgs showed some amino acid variations due to differences in virus genotype and subtype. To determine the immunogenicity of "a" determinant of recombinant sHBsAg, the sHBsAg sequence in the research was also examined for possibility of immune-escape mutant. The immune-escape mutant of HBV was known to have some rare substitution in amino acids residues in the "a" determinant region, such as G145R (Purdy *et al.* 2007), Q129R, and G145A (Koyanagi *et al.* 2000). Interestingly, one study also found a rare amino acid substitution Y161S located outside the "a" determinant region was an immune-escape mutant (Jinata *et al.* 2012). Based on multiple amino acid sequence alignment in the "a" determinant region of sHBsAg, the sequence used in this research was not identified as an immune-escape mutant (Fig 4).

The recombinant plasmid pHIPX4-sHBsAg was linearized in the AOX promoter region to facilitate its integration in the AOX locus of *H. polymorpha* NCYC495 (Saraya *et al.* 2012). Several papers have reported that the integration of sHBsAg in the *H. polymorpha* genome by the use of autonomously replicating sequence (HARS1) allowed higher integration frequency (Diminsky *et al.* 1997; Heijtink


Fig 4 Multiple amino acid sequence alignment in the "a" determinant region of sHBsAg.

*et al.* 2002; Bian *et al.* 2009).

The expression of sHBsAg in *H. polymorpha* NCYC495 transformants (S1 and S2) has been verified by diagnostic kit test and ELISA. Further analysis using Western blot showed that the recombinant sHBsAg appeared as a protein band at 19.8 kDa, which is similar with the purified sHBsAg used as a control (Fig 3B). The predicted molecular weight of sHBsAg calculated using ExPASy (Gasteiger *et al.* 2003) was 25.3 kDa. This molecular weight difference could be due to incomplete reduction of disulfide bonds in recombinant sHBsAg (Ottone *et al.* 2007). The presence of disulfide bonds will introduce a more compact shape of the proteins (Dunker and Kenyon 1976), hence faster migration rates of recombinant sHBsAg would be expected (Rath *et al.* 2009). Taken together, the *H. polymorpha* capable of expressing Indonesian sHBsAg developed in this work is an alternative source of recombinant HBsAg vaccine production.

## ACKNOWLEDGMENT

We thank Ida Van der Klei from University of Groningen for providing *Hansenula polymorpha* strain and expression vector. This research was partially funded by Ministry of Research, Technology and Higher Education for the National Hepatitis B Consortium.

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