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# Synthesis of hepatitis B virus surface protein derivates in yeast *S. cerevisiae*

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HBV surface proteins PreS1[13–59]-S, PreS1[20–59]-S, PreS1[30–59]-S, PreS1[40–59]-S, PreS1[50–59]-S, PreS1[90–119]-S were produced in *S.cerevisiae* and purified. Electron microscopy suggested spherical virus-like particle formation for all the proteins except PreS1[90–119]-S. The PreS1[90–119] sequence was demonstrated to decrease protein solubility. Proteins are suitable for *Tupaia* primary hepatocyte binding investigations, diagnostic products and vaccine candidate development.

**Key words:** hepatitis B virus, HBV surface proteins, preS1, virus-like particles

## INTRODUCTION

Hepatitis B virus (HBV) is a member of the family *Hepadnaviridae*. It causes transient and chronic liver infections in humans. Nearly 25% of chronic infections result in liver cancer [1]. Three HBV surface proteins share their carboxyterminal sequence. S (also known as HBsAg) has 226 amino acids, M has got additional 55 amino acids long preS2 domain, L also has 108 amino acids (ay subtypes) or 119 amino acids (ad subtypes) long preS1 domain. HBsAg forms spherical virus-like 22nm particles both in the plasma of infected persons and when produced in yeast [2]. It is used as a vaccine against HBV, however, non-responders and virus 'escape mutants' constitute a problem. PreS containing vaccines were suggested to be more effective. A vaccine consisting of virus-like particles containing full-length preS1, preS2 and S sequences was shown to be of superior immunogenicity when compared to HBsAg vaccines in mice and in clinical studies. In particular, a single dose of the vaccine elicited a successful immune response in 79% of subjects with inadequate response to previous immunisation, a significantly greater fraction than after a single dose of HBsAg vaccine [3]. The role of HBV surface protein in virus attachment and infectivity is being actively investigated. *Tupaia belangeri* primary hepatocytes are used for *in vitro* transfection studies [4]. Virus-like particle forming HBV surface protein construct PreS1[13–59][90–19]-S (amino acids according to ad subtype nomenclature) expressed in *S. cerevisiae* by us was used in a study by Prof. W. H. Gerlich and co-workers, which identified preS1 amino acids 13–59 as mediating attachment of the virus to its target cells [5].

The aim of the present work was to produce proteins containing S and variable length preS1 sequences, intended for future use in hepatocyte binding studies.

## MATERIALS AND METHODS

### Generation of yeast expression plasmids

HBV genotype D encoding plasmid HBV320 was provided by Prof. P. Pumpens [6]. Genes encoding HBV surface protein derivates incorporating whole HBsAg and variabale length N-attached preS1 polypeptides were constructed by PCR techniques. Genes encoding PreS1[13–59]-S, PreS1[20–59]-S, PreS1[30–59]-S, PreS1[40–59]-S, PreS1[50–59]-S, PreS1[90–119]-S were inserted separately into the XbaI site of the yeast expression vector pFD3 (variant of pFX7) under control of hybrid *GAL10-PYK1* promoter [7] and sequenced. Yeast *Saccharomyces cerevisiae* strain FH4C was transformed separately with plasmids pFD3-PreS1[13–59]-S, pFD3-PreS1[20–59]-S, pFD3-PreS1[30–59]-S, pFD3-PreS1[40–59]-S, pFD3-PreS1[50–59]-S, pFD3-PreS1[90–

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119]-S, with pFX7 as a negative control and with pFX7-S encoding HBsAg. The transformants were selected for their viability in the presence of formaldehyde.

**Expression and purification of recombinant proteins**
Protein purification was based on the experience with other recombinant HBV surface proteins. Yeast cells were grown in YEPD medium (yeast extract 1%, peptone 2%, glucose 2%) supplemented with 8 mM formaldehyde at 28 °C for 24 h. Protein expression was induced by adding galactose to a final concentration of 2.5%. Yeast cells were harvest-ed after 16 h and disrupted using glass beads (0.5 mm, Sigma) in disruption buffer (0.01M phosphate buffer pH 7.2, 1 mM EDTA, 2 mM PMSF). The supernatants were collected after centrifugation at 700 *g* for 4 min at 4 °C, the pellets were resuspended in disruption buffer, centrifugation was repeated. The supernatants were centrifugated at 7830 *g* for 40 min. The pellets were resuspended in disruption buffer containing 0.5% Tween-20 and incubated at 0 °C for 1 h with constant rocking. After centrifugation at 6340 *g* for 20 min the pellets were resuspended in disruption buffer containing 1% Tween-20, the incubation and centrifugation were repeated. SDS-PAGE was used to identify recombinant proteins. CsCl was dissolved in 1% Tween-20 containing supernatants to the final concentration of 250 g/l. In case of insoluble protein, a resuspended pellet was transferred on 250 g/l CsCl solution. The solutions were centrifuged at 100000 *g* for 40 h. Fractions containing recombinant HBV proteins were identified by SDS-PAGE, pooled, diluted with CsCl solution of 1.2 g/cm$^3$ density and re-centrifuged. Recombinant protein containing fractions were dialysed against PBS.

**SDS-PAGE and immunoblot**
Protein samples were separated on 12% SDS-PAGE gels and transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, USA) by semi-dry blotting. The membrane was blocked in TBS-T (20 mM Tris-HCl, 8 g/l NaCl, pH 7.6, 0.1% Tween-20) and incubated with HBsAg specific monoclonal antibodies HB1 (A. Žvirblienė, Institute of Biotechnology, Li-thuania) diluted 1:2000 in TBS-T and thereafter, with anti-mouse IgG-HRP conjugate (Sigma-Aldrich, Steinheim, Germany), diluted 1:1000 in TBS-T. The membrane was stained by 4-chloro-naphthol supplemented with $H_2O_2$ (Sigma, Steinheim, Germany).

Proteins on SDS-PAGE gels were visualized by silver-staining.

**Electron microscopy**
CsCl gradient fractions containing recombinant proteins were placed on 400-mesh carbon-coated palladium grids. The samples were stained with 2% aqueous uranyl acetate solution and examined using a JEM-100S electron microscope.

## RESULTS AND DISCUSSION

Genes encoding HBV surface proteins PreS1[13–59]-S, PreS1[20–59]-S, PreS1[30–59]-S, PreS1[40–59]-S, PreS1[50–59]-S, PreS1[90–119]-S were constructed and separately cloned into the yeast expression vector pFD3. Recombinant protein synthesis was induced in the transformed yeast and protein purification steps were carried out. Proteins of expected molecular mass (32.0 kDa, 30.5 kDa, 29.3 kDa, 28.3 kDa, 27.0 kDa and 29.9 kad, respectively) were detected by SDS-PAGE. PreS1[13–59]-S, PreS1[20–59]-S, PreS1[30–59]-S, PreS1[40–59]-S, PreS1[50–59]-S were most abundant in 1% Tween-

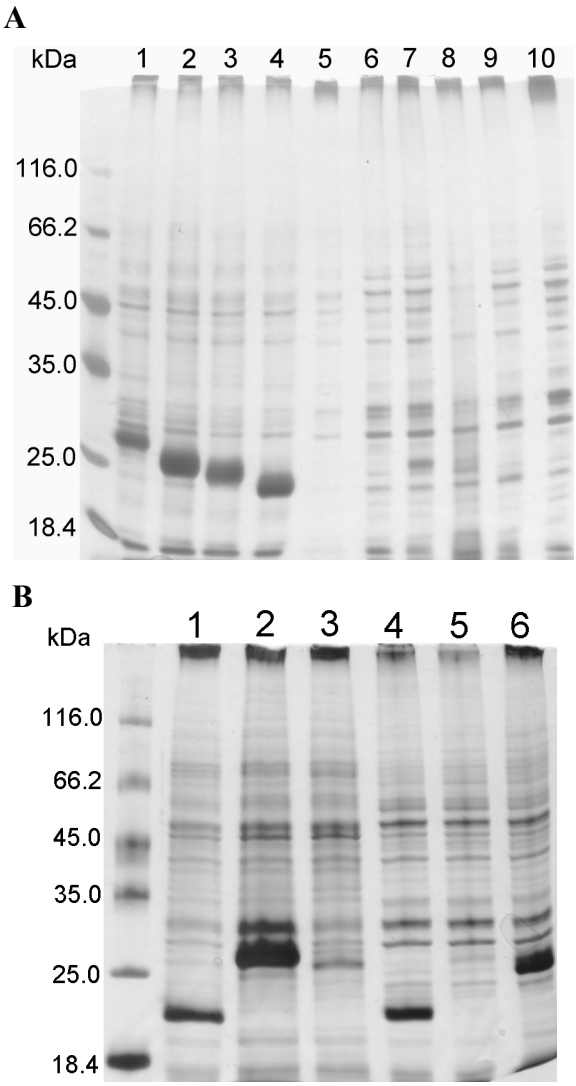


**Fig. 1.** Purification of recombinant HBV protein
(A) 1–5: 1% Tween-20 extracts; 6–10: pellet after extraction with 1% Tween-20.
Protein Molecular Weight Marker (Fermentas, Vilnius, Lithuania); lanes 1, 6: PreS1[20–59]-S; 2, 7: PreS1[30–59]-S; 3, 8: PreS1[40–59]-S; 4, 9: PreS1[50–59]-S; 5, 10: pFX7 transformed yeast samples.
(B) 1–3: 1% Tween-20 extracts; 4–6: pellet after extraction with 1% Tween-20.
1, 4: HBsAg; 2, 5: PreS1[13–59]-S; 3, 6: PreS1[90–119]-S.

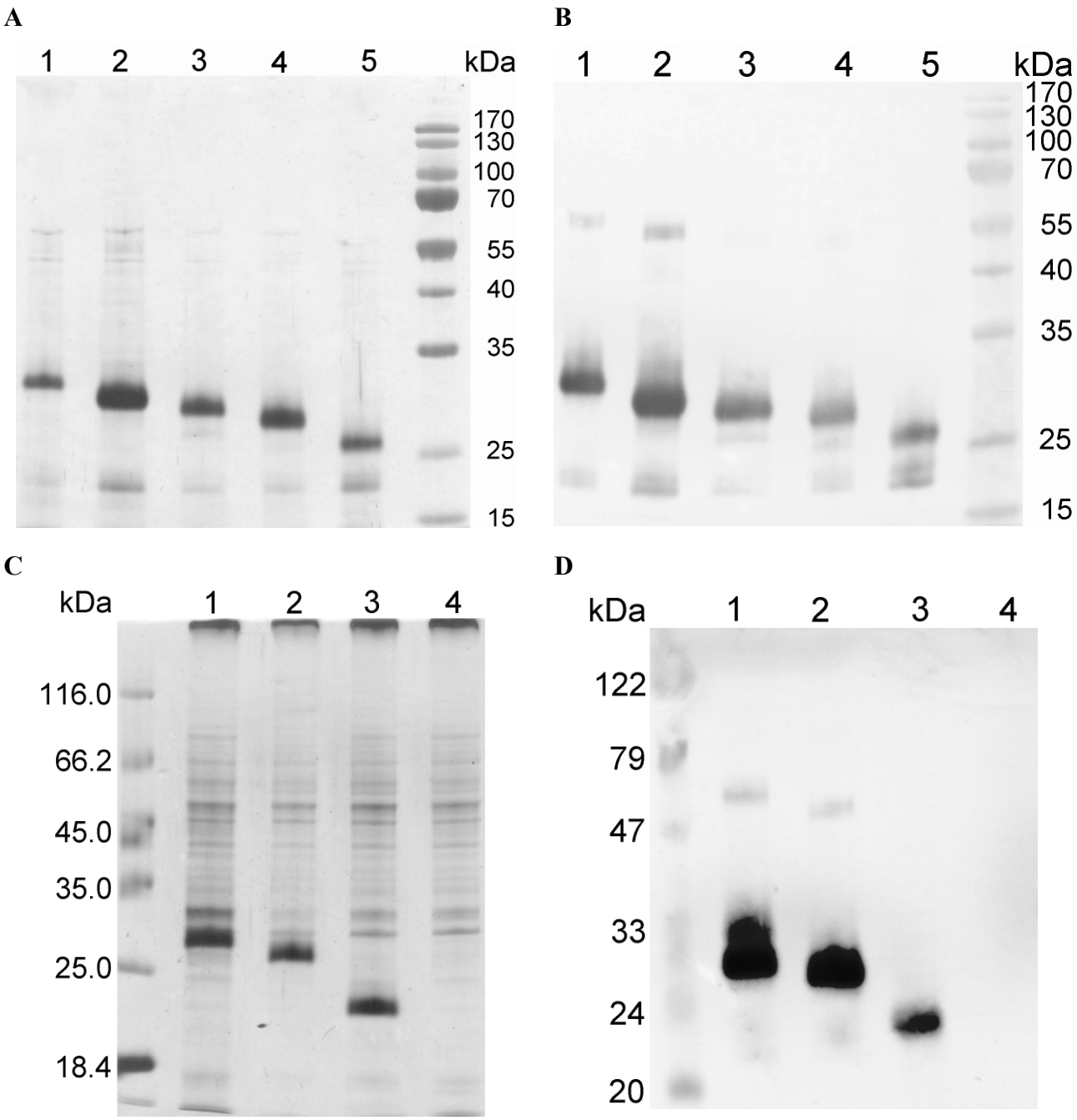


**Fig. 2.** Immunodetection of recombinant HBV proteins
(A) SDS-PAGE of purified proteins, (B) immunoblot with anti-HBsAg specific HB1 antibody:
1 – PreS1[20–59]-S, 2 – PreS1[30–59]-S, 3 – PreS1[40–59]-S, 4 – PreS1[50–59]-S, 5 – HBsAg; PageRuler™ Prestained Protein Ladder (Fermentas, Vilnius, Lithuania).
(C) SDS-PAGE of 1% Tween-20 extracts; (D) Immunoblot with anti-HBsAg specific HB1 antibody:
1 – PreS1[13–59]-S, 2 – PreS1[90–119]-S, 3 – HBsAg; 4 – yeast FH4C, Protein Molecular Weight Marker or Prestained Protein Molecular Marker (Fermentas, Vilnius, Lithuania).

20 extracts as revealed by SDS-PAGE, but almost all PreS1[90–119]-S was found in insolubilized sediment (Fig.1). For comparison, HBsAg was partly solubilized by 1% Tween-20, indicating PreS1[90–119] to decrease solubility while the test PreS1[N-59] sequences increase it. Protein solubility under specific conditions allowed establishing an effective purification protocol. Proteins from 1% Tween-20 extracts were concentrated at approximately 1.16 g/cm$^3$ by CsCl density gradient centrifugation. Protein yield from 1 l yeast culture was approximately 19.4 mg/l, 6.6 mg, 16.8 mg, 7.6 mg, 7.6 mg, 6.1 mg/l (0.85 mg/l extracted with 1% Tween and 5.2 mg/l insoluble) for PreS1[13–59]-S, PreS1[20–59]-S, PreS1[30–59]-S, PreS1[40–59]-S, PreS1[50–59]-S, PreS1[90–119]-S, respectively. Their identity was confirmed by immunoblot with HBsAg specific monoclonal

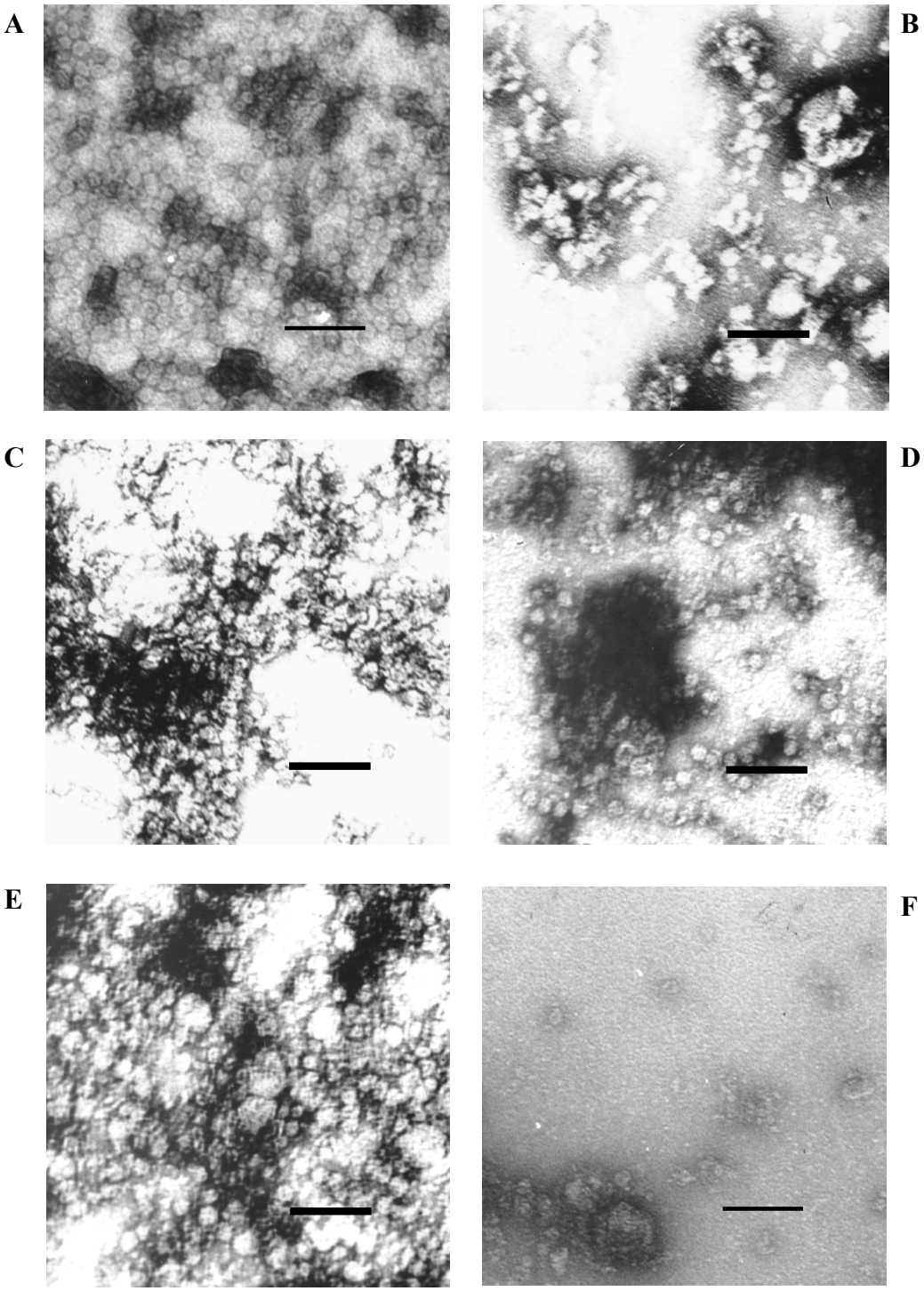


**Fig.3.** Electron microscopy of recombinant HBV proteins (black bar corresponds to 100 nm) (A) PreS1[13–59]-S, (B) PreS1[20–59]-S, (C) PreS1[30–59]-S, (D) PreS1[40–59]-S, (E) PreS1[50–59]-S, (F) PreS1[90–119]-S.

antibody (Fig. 2). Electron microscopy suggested that all the proteins, except PreS1[90–119]-S, formed ~22 nm spherical virus-like particles (Fig. 3). Particles of similar size are formed by HBsAg isolated from blood serum and by *S. cerevisiae* expressed HBsAg [2], PreS1[13–59][90–19]-S [5], indicating PreS1[90–119] to hinder particle formation and decrease solubility while PreS1[13–59] restores them.

## CONCLUSIONS

Variable N-terminal length preS1-S proteins were successfully produced in *S. cerevisiae* and are suitable for hepatocyte binding investigations, diagnostic products and vaccine candidate development. PreS1[90–119] was demonstrated to decrease protein solubility and hinder particle formation. The PreS1[N-59] sequences tested increased solubility and virus-like particle formation.

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## HEPATITO B VIRUSO PAVIRŠIAUS BALTYMŲ DARINIŲ SINTEZĖ MIELĖSE *S. CEREVISIAE*

S a n t r a u k a

Hepatito B viruso paviršiaus baltymai PreS1[13–59]-S, PreS1[20–59]-S, PreS1[30–59]-S, PreS1[40–59]-S, PreS1[50–59]-S, PreS1[90–119]-S susintetinti *S. cerevisiae* mielėse ir išgryninti. Baltymų elektroninė mikroskopija rodo, jog jie, išskyrus PreS1[90–119]-S, formuoja sferines, į virusą panašias daleles. PreS1[90–119] seka sumažina baltymų tirpumą. Baltymus galima panaudoti tiriant sąveiką su tupajų pirminiais hepatocitais, kuriant diagnostikos priemones, vakcinas.