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Highly efficient production of phosphorylated hepatitis B core particles in yeast *Pichia pastoris*

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Virus-like particles (VLPs) of the recombinant hepatitis B virus (HBV) core protein (HBc) are routinely used in HBV diagnostics worldwide and are of potential interest as carriers of foreign peptides (e.g., immunological epitopes and targeting addresses, and/or as vessels for packaged diagnostic and therapeutic nanomaterials). Despite numerous reports exploiting different expression systems, a rapid and comprehensive large-scale methodology for purification of HBc VLPs from yeast is still lacking. Here, we present a convenient protocol for highly efficient production and rapid purification of endotoxin-free *ayw* subtype HBc VLPs from the methylotrophic yeast *Pichia pastoris*. The HBc gene expression cassette along with the geneticin resistance gene was transferred to the *P. pastoris* genome via homologous recombination. A producer clone was selected among 2000 transformants for the optimal synthesis of the target protein. Fermentation conditions were established ensuring biomass accumulation of 163 g/L. A simple combination of pH/heat and salt treatment followed by a single anion-exchange chromatography step resulted in a more than 90% pure preparation of HBc VLPs, with a yield of about 3.0 mg per 1 g of wet cells. Purification is performed within a day and may be easily scaled up if necessary. The quality of HBc VLPs was verified by electron microscopy. Mass spectrometry analysis and direct polyacrylamide gel staining revealed phosphorylation of HBc at at least two sites. To our knowledge, this is the first report of HBc phosphorylation in yeast.

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Introduction

Hepatitis B virus (HBV)[1] from the *Hepadnaviridae* family is a major cause of human liver disease, resulting in approximately 620,000 deaths worldwide each year [1]. About 4.5 million new HBV infections occur each year, of which a quarter progress to liver disease [2]. The virus is composed of an outer envelope containing surface proteins integrated in a lipoprotein shell and an inner nucleocapsid assembled from the HBV core protein (HBc) that encloses the viral genomic DNA and the viral polymerase [3]. Virtually all HBV infected individuals can produce high titers of an anti-HBc antibody, which is one of the most specific serological markers of past (anti-HBc IgG) or current (anti-HBc IgM) HBV infections [4].

Heterologously expressed HBc spontaneously assembles into the virus-like particles (VLPs) that are routinely used for HBV diagnostics worldwide [5]. Because HBc is known to induce strong B-cell, T-cell, and cytotoxic T-cell responses in hepatitis B patients [6] in both a T-cell dependent and T-cell independent manner [7], it might be regarded as a component of a novel prophylactic and/or therapeutic HBV vaccine [8]. The ability of HBc carrier to provide inserted epitopes with T-cell help [9] and preferential priming of Th1 cells, without any requirement for adjuvants [10], stimulates the development of a broad range of vaccine prototypes on the basis of HBc VLPs [8]. Recently, HBc has attracted special interest in medicinal nanotechnology as a putative packager of organic and inorganic compounds, including stimulatory oligonucleotides, low molecular weight drugs, and magnetic particles.

Yeast systems have been used extensively for expression of a large number of structural genes from many mammalian viruses, which results in a formation of naturally folded VLPs [11–16]. This has led to a generation of licensed prophylactic vaccines against human HBV and papillomaviruses [8,17]. One of the most prominent yeast expression systems is based on the methylotrophic *Pichia pastoris* strain, which is used successfully to produce more than 500 proteins both for basic laboratory research and industrial manufacturing [18,19]. Although expression of the *HBc* gene in yeast cells including *P. pastoris* have been described by several authors [20–24], the majority of published HBc VLP purification methods remain either too complicated and time-consuming or

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[1] *Abbreviations used:* AEC, anion-exchange chromatography; CBB, Coomassie Brilliant Blue; HBV, hepatitis B virus; HBc, HBV core protein; PAAG, polyacrylamide gel; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VLP(s), virus-like particle(s).

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non-effective due to the high costs and/or low output of the target protein.

Naturally folded HBc, either isolated from human tissues or recombinantly expressed in mammalian cells, appears as a phosphoprotein [25,26], being phosphorylated on serine residues at the carboxy-terminal part of the molecule [27]. In contrast, phosphorylation has not been detected for *Escherichia coli*- and yeast-derived HBc [28]. It has been demonstrated that HBc phosphorylation plays an important role in HBV replication and capsid localization [29,30]. In the present work, we establish the efficient expression of the *HBc* gene in *P. pastoris* and develop a rapid purification method for HBc VLPs. In contrast to previous reports, HBc is found to be phosphorylated at at least two sites within the molecule. We suggest such yeast-derived HBc VLPs are of value for diagnostic purposes and vaccine development as well as for HBV replication studies.

Materials and methods

Construction of an expression vector and selection of clones

The *HBc* gene from plasmid pHB320 containing the full HBV genome, genotype D, subtype *ayw* (GenBank Accession No. X02496; [31]), was PCR-amplified and ligated into the *Bam*HI/*Eco*105I-treated vector pPIC3.5K (Invitrogen), under control of the *AOX1* promoter. After sequencing, the resulting pPIC-HBc plasmid was linearized with *Ecl*136II in *AOX1* promoter region and used for transformation of the *P. pastoris* GS115 *his4* strain by electroporation. Mut^+His^+ transformants were isolated on agarized minimal medium (1.34% yeast nitrogen base, 2% glucose, 4×10^{-5}% biotin, 2% agar). For selection of clones with multiple integration units, transformants were replica-plated onto yeast extract peptone dextrose (YEPD) agarized medium containing increased concentrations of the G418 antibiotic (1.8–2.5 mg/mL).

Southern blotting

To estimate the approximate expression cassette copy number in *P. pastoris*, chromosomal DNA from selected transformants was digested with *Bgl*II and separated by agarose gel electrophoresis. After transferring to a nitrocellulose membrane, DNA was hybridized with a biotin-labeled *HIS4*-specific probe (~600-bp *Kpn*I restriction fragment from the pPIC3.5K) using the Biotin DecaLabel™ DNA Labeling Kit. The reaction was further processed and developed by the Biotin Chromogenic Detection Kit. All the enzymes, protein and DNA molecular weight (MW) markers as well as kits used in our study were purchased from Fermentas (Vilnius, Lithuania) unless otherwise indicated.

Cultivation in flasks

Induction of *HBc* gene expression in *P. pastoris* was achieved according to the protocol of the manufacturer with the following minor modifications: selected clones were incubated at 30 °C on a shaker in 0.5-L Erlenmeyer flasks containing 100 mL of buffered complex glycerol medium (BMGY) for 20–24 h until OD_{590} 6–8; cells were then harvested by low-speed centrifugation and resuspended in the same volume of buffered complex methanol medium (BMMY) containing 0.5% methanol. Each subsequent day, 100% methanol was added to a final concentration of 0.5%, and cells were harvested 3 days after induction.

The level of HBc production was estimated by disrupting 20 optical units of yeast cells by 425–600-μ glass beads (Sigma) in 200 μL of 20 mM Tris–HCl, pH 8.0, eight times for 0.5 min. Debris was separated by low-speed centrifugation, and the supernatant was serially diluted for an immunodiffusion assay [32] using a polyclonal rabbit anti-HBc antibody (obtained after immunization with *E. coli*-derived HBc VLPs).

Large-scale cultivation

A volume of 500 mL of seed material was used to inoculate 4.5 L of BMGY in a fermentor. Seed material was prepared in two steps: stock culture from storage at −80 °C was plated on agarized minimal medium, and a single colony was inoculated in 5 ml of YEPD and cultured at 30 °C for 48 h. A volume of 0.1 mL of this primary seed material was transferred to each of five 0.5-L flasks containing 100 mL of BMGY and 10 mg/L chloramphenicol, and flasks were incubated at 250 rpm at 30 °C for 20–24 h to a final OD_{590} 6.0–8.0.

Fermentation conditions: A 10-L fermentor (Bioflo 410, New Brunswick Scientific) was filled with 4.5 L BMGY (with glycerol concentration 40 g/L) and 0.5 L seed material in BMGY. Non-enriched air was used throughout the fermentation. The dissolved oxygen was set at 20%, the aeration rate on the first day was up to 1 vol/min, and the stirring speed was up to 1200 rpm, while the incubation temperature was 30 °C, and the pH was controlled with 25% (v/v) NH_4OH to keep the pH above 5.0. After the glycerol exhaustion at 18 h, 20 mL of 50% glycerol and 10 mL of 100% methanol was added. One hour later, the methanol supply was set at a rate of 3.0 mL/L/h, and the air supply was increased to 5 vol/min. Cultivation continued for 92 h with methanol feeding adjusted to a consumption rate of 6.8 g/L/h. Antifoam M30 (Serva) was used to prevent extensive foam formation. Cells were harvested at 3000*g* for 10 min at 4 °C. After washing once with dH_2O, the cell pellet was stored at −80 °C until use.

Purification of HBc VLPs

A 4-g portion of the frozen yeast cells was resuspended in 16 mL of lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF) and disrupted with a French press (3 cycles, 20,000 psi). The soluble fraction was separated by centrifugation for 15 min at 15,500*g* and the pH was adjusted to 8.0 with 0.5 M NaOH. The supernatant was incubated for 1 h at +65 °C and subsequently centrifuged for 25 min at 15,500*g*. Solid ammonium sulfate was then added to the supernatant to 40% saturation, which was incubated for 0.5 h at 4 °C and centrifuged again for 25 min at 15,500*g*. The sediment was dissolved in a minimal amount (1 mL) of phosphate buffer containing 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Triton X-100, pH 7.4, and loaded onto a pre-packed anion-exchange HiPrep 16/10 DEAE Fast Flow column (20-mL bed volume) connected to an ÄKTA chromatography system (Amersham Biosciences). The column was preequilibrated with phosphate-buffered saline, pH 7.4 (Sigma), and run at 5 mL/min. Column-bound proteins were eluted by a linear gradient with phosphate buffer containing 1 M NaCl.

E. coli-derived HBc VLPs used as a control were purified essentially as described previously [33].

Analytical methods

Protein samples were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), with a 4% stacking and 15% separating polyacrylamide gel (PAAG), according to standard protocols. To visualize protein bands, the gels were stained with Coomassie Brilliant Blue (CBB) G-250. Alternatively, separated proteins were transferred onto nitrocellulose membranes and detected by immunoblotting with the monoclonal anti-HBc antibody 13C9 [34] and the anti-mouse IgG peroxidase conjugate (Sigma).

Protein samples were subjected to native 1% agarose gel electrophoresis in TAE buffer (pH 8.4) for about 0.5 h at 5 V/cm. Nucleic

acids in agarose gels were visualized by ethidium bromide staining. For determination of protein concentration, a Bradford assay [35] was used, and the purity of HBc samples was estimated by densitometric analysis of the CBB-stained PAAG.

The endotoxin level in the protein samples was determined by a Limulus amoebocyte lysate (LAL) Pyrogent® Plus test kit according to the manufacturer's protocol (BioWhittaker, Walkersville, MD).

To detect phosphorylated proteins, PAAG was stained directly with the ProQ® Diamond phosphoprotein gel stain (Invitrogen) according to the manufacturer's instructions. Full-length HBc molecules as well as their C-terminal domains resulting from proteinase K cleavage were analyzed. For the proteinase K reaction, VLPs were treated for 10 min at 95 °C in buffer containing 1% SDS and 2% β-mercaptoethanol followed by the addition of proteinase K and incubation for 5 min at 50 °C.

For electron microscopy, the protein samples were adsorbed on carbon–formvar-coated copper grids and negatively stained with 1% uranyl acetate aqueous solution. The grids were examined with a JEM-1230 electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

For whole protein analysis in mass spectrometry, 2 μL of purified VLPs at 1 mg/mL concentration in 20 mM Tris–HCl, pH 8.0, was mixed with 2 μl 10% trifluoroacetic acid and 2 μl DHAP. For tryptic digestion of proteins, the CBB-stained band was excised from SDS–PAAG, incubated for 2 × 1 h in 0.2 M ammonium bicarbonate and 50% acetonitrile at 30 °C and incubated for 2 × 20 min in 100% acetonitrile at room temperature. A volume of 0.1 mg/mL trypsin (Sigma, proteomics grade) in 1 mM HCl was mixed 1:1 with 50 mM ammonium bicarbonate in 10% acetonitrile. Gel pieces were covered with the trypsin solution so that the gel absorbed approximately two thirds of the solution's volume. The gel was further incubated for 3 h at 30 °C. A volume of 2 μL of buffer covering the gel pieces was mixed with 2 μL 10% trifluoroacetic acid and 2 μl DHAP solution (15 mg/mL DHAP in 75% ethanol, 2.5 mM diammonium hydrogen citrate). All samples were analyzed on a Bruker Daltonics Autoflex MALDI-TOF mass spectrometer.

Results and discussion

Selection and cultivation of the HBc producer strain

The yeast expression system was chosen due to its relatively simple fermentation design and high final cell densities. In addition, data from other groups indicate that *P. pastoris*-derived HBc preparations are superior to *E. coli*-derived HBc VLPs in anti-HBc antibody diagnostic assays [23,24]. Additionally, yeast products do not harbor pathogens, viruses, or pyrogens.

It has been shown previously that the expression level in *P. pastoris* VLP producers can be substantially increased by extensive screening for clones with multicopy insertions of expression cassettes [15,23,36,37]. For this, about 2000 Mut$^+$His$^+$ transformants were screened for their resistance level to increased doses of the G418 antibiotic as a selection marker. The majority of the clones were resistant to G418 at concentrations below 1.8 mg/mL, while 1–2% of clones were able to grow at concentrations up to 2.5 mg/mL, indicating a possible multiple-insertion event. Clones with increased resistance generally exhibited higher though variable synthesis of the target protein (Fig. 1a) which correlated well with their anti-HBc titers in immunodiffusion: highly resistant clones reacted at dilutions up to 1:128, while control clones with single-copy inserts reacted at a dilution 1:8 (data not shown). In this way, clone E1, with the highest production level confirmed by SDS–PAGE (Fig. 1a, lane 4), was selected.

To verify multiple-target gene insertion events in clones with increased resistance to G418, samples of chromosomal DNA from individual clones were subjected to Southern blot analysis (Fig. 1b). This analysis revealed the presence of a 2.7-kb fragment corresponding to the chromosomal *HIS4* gene and a larger, approximately 6.0-kb fragment corresponding to the expression cassette integrated at the *AOX1* locus. As expected, a putative single-copy transformant exhibited two bands of similar intensity (Fig. 1b, lane 2), whereas in clones with an increased resistance, the 6.0-kb band dominated over the 2.7-kb band, suggesting a multiple-insertion pattern (Fig. 1b, lanes 3–7). Notably, clones with apparently even more copies than E1 (e.g., clone E) still produced remarkably less HBc (compare Fig. 1a and b, lanes 3 and 4). Although the exact insert copy number was not detected, this finding indicates a certain optimal level of target gene dosage. This is consistent with data for expression of other viral structural genes in *P. pastoris* (e.g., maximal synthesis of measles virus nucleoprotein was detected in a transformant with 10 copies of the target gene, whereas a further increase in the gene copy number led to reduced expression [15]). Our data clearly show that the expression potential of the *P. pastoris* expression system could sometimes be underevaluated by limited screening of clones.

Clone E1 was cultivated in a fermentor as described in the Materials and methods section, with an HBc expression level of 2–3% of the total cell proteins at the end of cultivation. Although this was about three times lower than the HBc level obtained with the *E. coli* expression system [38,39], it was high enough to establish an efficient and short purification protocol ensuring about 90% purity of the target protein (discussed below). For our fermentation, we did not use oxygen-enriched air, which has often been used in *P. pastoris* fermentation systems [23,40]. The final biomass yield under these conditions was 163 g/L (an average from two independent cultivations); with oxygen enrichment, it was possible to obtain twice the amount of biomass, but with a three times lower HBc synthesis level (data not shown). Although more fermentation experiments are needed to make definite conclusions about the effect of oxygen, we propose that slower biomass accumulation is beneficial for HBc synthesis.

Purification of HBc VLPs

To make purification cost-effective and fast, we aimed to avoid many commonly used protein purification steps such as centrifugation in a sucrose gradient, affinity and size-exclusion chromatography, filter-concentration, and dialysis. Disrupting yeast cells with the French press is highly reproducible and easy to scale-up. The majority of the HBc was found in the soluble fraction (Fig. 2a, lane 1), though a strong degradation pattern was detectable by a Western blot (Fig. 2b, lane 1). A heat treatment has been used by several groups as a purification step applicable for both bacterial and yeast-derived HBc preparations [22,41,42]. In addition, Naito et al. [41] demonstrated that contaminating proteins were removed from bacterial lysates more efficiently at pH 6.0 rather than at neutral pH. Under our conditions, heat treatment of yeast lysates at pH 6.2 (this was the pH of the non-adjusted crude cell lysate) precipitated the majority of HBc, while a rise in the pH significantly increased the solubility of HBc, with pH 8.0 being optimal for recovery of the target protein (Fig. 2a, and data not shown). Further experiments revealed that a heat treatment at pH 8.0 of either the supernatant or crude cell homogenate resulted in nearly identical HBc recovery (data not shown), enabling the exclusion of the initial centrifugation step from the protocol. Moreover, heat treatment led to precipitation of degraded forms of HBc (Fig. 2b, lanes 1 and 2). We suggest that most HBc degradation products are not incorporated in the capsid structure and therefore are thermally less stable than VLPs. Altogether, our data demonstrate the effect

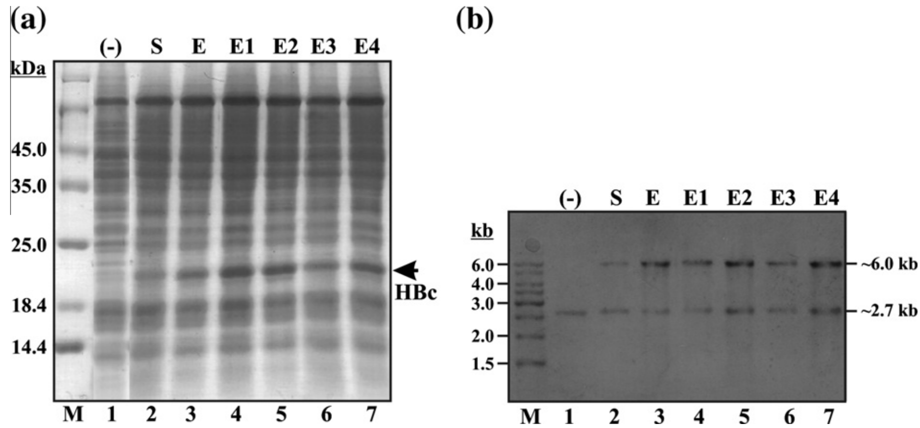

Fig. 1. Correlation between *HBc* gene expression level and the amount of integration units in individual *P. pastoris* subclones estimated by CBB-stained PAAG (a) and Southern blotting (b). Lane 1, negative control, non-transformed *P. pastoris* cells. Lanes 2–7, *P. pastoris* clones harboring single (S; lane 2) and multiple (E–E4; lanes 3–7) *HBc* gene insertions. Only a part of the multicopy clones is shown to demonstrate deviation in expression level. M, protein (a) and DNA (b) MW standards.

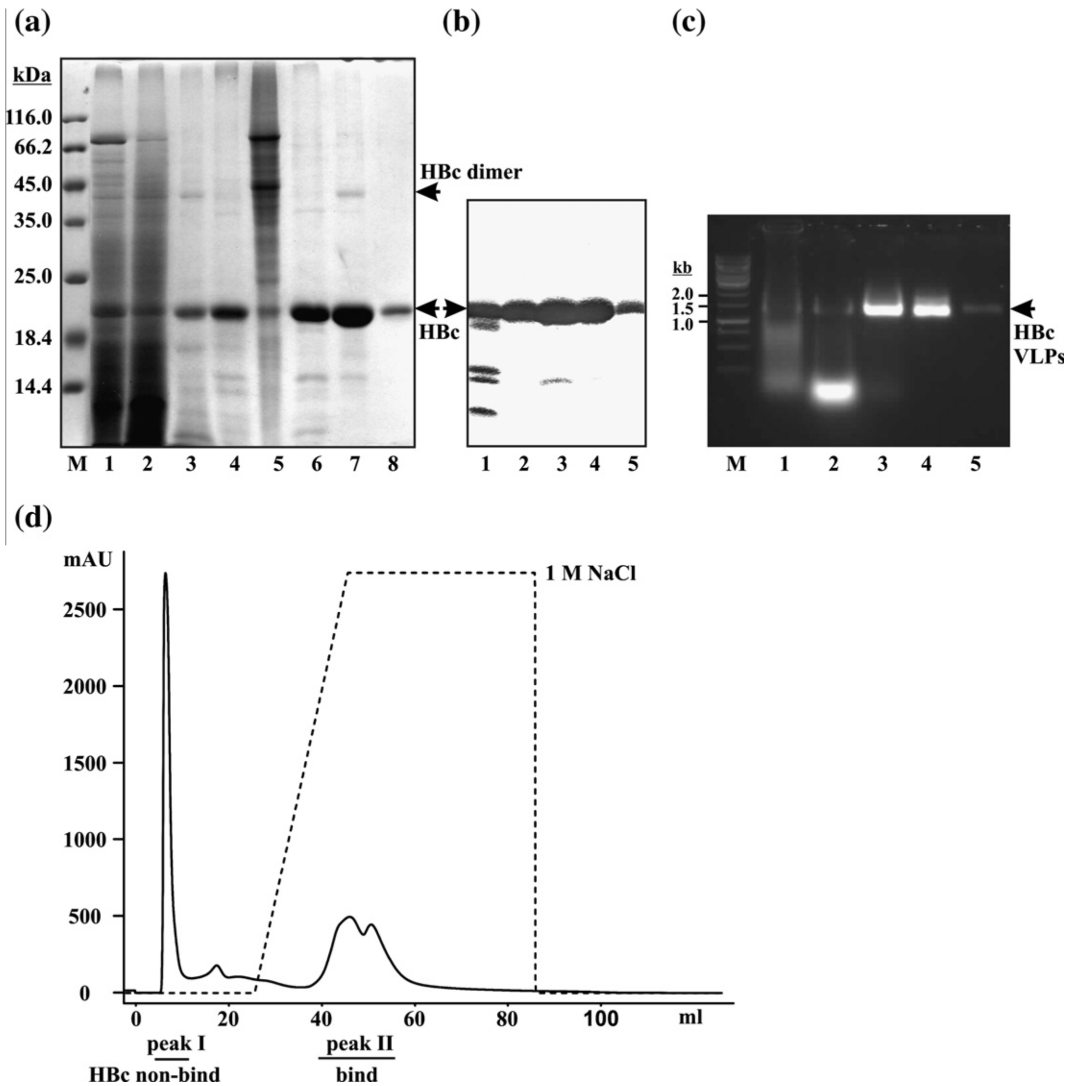

Fig. 2. Main purification steps for HBc VLPs. (a) CBB-stained PAAG illustrating HBc purity in protein samples. Lanes 1 and 2, soluble and unsoluble fractions, respectively. Lanes 3 and 4, soluble fraction after heat treatment at pH 6.2 and 8.0, respectively. Lane 5, heat-precipitated proteins from supernatant at pH 8.0. Lane 6, ammonium sulfate precipitate. Lanes 7 and 8, proteins from AEC peaks I and II, respectively. (b) and (c) Western blotting and native agarose gel electrophoresis, showing HBc degradation pattern and presence of nucleic acids, respectively. Lanes 1 and 2, non-treated and heat-treated cell supernatants, respectively. Lane 3, dissolved ammonium sulfate precipitate. Lanes 4 and 5, proteins from AEC peaks I and II, with column flow-through and bound material, respectively (d). M, protein (a) and DNA (c) MW standards.

of the pH during the heat treatment procedure, although it is clear that the optimal pH value should be determined for each individual expression event.

Ammonium sulfate precipitation has been routinely used in HBc VLP concentration and purification protocols [22,33,43]. Under our conditions, a 0.5-h incubation was enough to precipitate more than 90% of the HBc from solution, with further successful and complete solubilization of the precipitate (data not shown). This procedure also removed most of the non-specific nucleic acids from the HBc preparation (Fig. 2c). Thus, a simple combination of pH/heat treatment followed by salt precipitation effectively enriched the concentration of the HBc in solution (Fig. 2a, lane 6).

Size-exclusion chromatography and/or sucrose gradient ultracentrifugation are often recommended as final steps in HBc purification [23,24,44]. These methods, however, strongly limit sample volume and increase costs. We looked to ion exchangers as a relatively cheap, robust material able to withstand harsh cleaning-in-place conditions, typically with sodium hydroxide. Other researchers have reported recovery of *E. coli*-derived HBc VLPs from weak anion exchange matrices such as DEAE Sephacel or Streamline DEAE [43,45]. We subjected the enriched HBc solution to DEAE Sepharose with similar characteristics and scalability possibilities. According to the anion-exchange chromatography (AEC) profile, proteins were separated into two dominant peaks representing column-bound and non-bound material (Fig. 2d). SDS–PAGE revealed that both peaks contain target protein, though with strong predominance of HBc in peak I (Fig. 2a, lanes 7 and 8). Thus, under conditions described, the majority of the HBc material did not bind to the matrix and was eluted as a sharp peak within the column void volume (between 5 and 8 mL). However, about 10% of the HBc was retained on the column and was eluted only at increased salt concentrations.

Characterization of HBc VLPs

As detected by SDS–PAGE, purified HBc migrated in PAAG according to its calculated MW, namely, 21.1 kDa, and showed no visible degradation in Western blotting (Fig. 2a, lane 7 and b, lane 4). Despite the presence of some minor bands of contaminants visible in overloaded PAAGs, the final purity of the HBc might be roughly estimated as at least 90%. The output of HBc VLPs reached 3 mg from 1 g of wet cells or about 700 mg from 1 L of initial *P. pastoris* fermentation culture. For comparison, reported yields by others who used *P. pastoris*-driven *HBc* gene expression were 69 and 64 mg/L, respectively [23,24]. Our greater output might be a consequence of a more efficient producer strain and/or an optimized purification protocol. In addition, one can speculate that different HBV genotypes (*adw* or *ayw* used in these or our studies, respectively) can influence the yield due to different behaviors of the target protein during purification.

The presence of nucleic acids in HBc VLPs was detected by agarose gel electrophoresis, where particles migrated along with the 1.5-kb dsDNA band (Fig. 2c). These VLP-associated nucleic acids can be eliminated by RNase (but not DNase) treatment (data not shown), confirming that heterologously expressed HBc VLPs predominantly encapsidate host-derived RNA [23,46]. To obtain empty RNA-free HBc VLPs for *in vivo* applications, Broos et al. [43] used combined Mono Q and Heparin column chromatography, though the yield was rather low. Alternatively, we and others have demonstrated that under low ionic strength, VLP-associated RNA can be substituted by short, defined DNA fragments [33,47]. Moreover, potential for controlled dis- and re-assembly makes VLPs especially attractive for gene and drug delivery applications [48,49]. The latter approach is also of value for the removal of non-specific nucleic acids from VLP preparations.

For further characterization of the final product, we subjected protein from both AEC peaks to electron microscopy. The non-bound fraction appeared as a heterogeneous mixture of correctly folded icosahedral $T = 3$ and $T = 4$ particles, with a predominance of the larger $T = 4$ form (Fig. 3a). These VLPs exhibited similar morphology to the authentic HBc particles derived from an HBV-infected liver as well as to recombinant VLPs obtained from bacterial cells expressing the *HBc* gene [50,51]. Interestingly, column-bound material also contained assembled VLPs, but these particles tended to aggregate possibly due to their association with remaining impurities (Fig. 3b). Thus, the final chromatography step improved not only the purity, but also the homogeneity of the VLP preparation.

The endotoxin level in the final product was less than 100 EU/mg of pure protein, which makes it attractive both for *in vitro* and *in vivo* applications. It should be noted that the low endotoxin level was achieved with relatively low synthesis of HBc VLPs compared to *E. coli* expression systems (data not shown).

The length of yeast-expressed HBc was investigated by MALDI-TOF mass spectrometry. The MW of full-length HBc theoretically is 21,116 Da (or 20,985 Da without the first methionine). Our data indicated that the MW of yeast-derived HBc is 21,324 Da (Fig. 4a). In parallel, we determined the MW of the same protein produced in *E. coli* to be 21,136 Da. As discussed in Watelet et al. [23], *E. coli*-produced HBc has its first *N*-acyl-methionine preserved, thus the theoretical MW is 21,144 Da, which is close to our observed data and well within the instrument precision for the given MW. However, the MW of the yeast-produced HBc was significantly higher than the theoretical value, and the difference could not be explained solely by instrument error. Therefore, we assumed that the increase in MW is due to some post-translational modification that does not occur in *E. coli*. Additionally, the protein peak in the mass spectrometer was significantly wider in the case of the yeast-expressed protein, suggesting that the material might

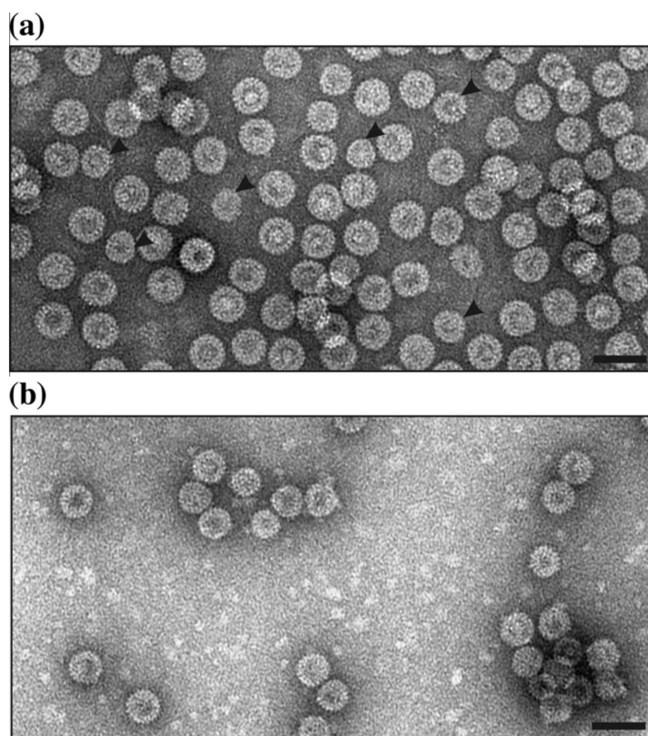

Fig. 3. Electron microscopy of purified HBc VLPs from AEC eluted in column void volume (a), and at an increased salt concentration (b). Some of the smaller $T = 3$ quasi-symmetry particles are marked by arrowheads. Scale bar: 50 nm.

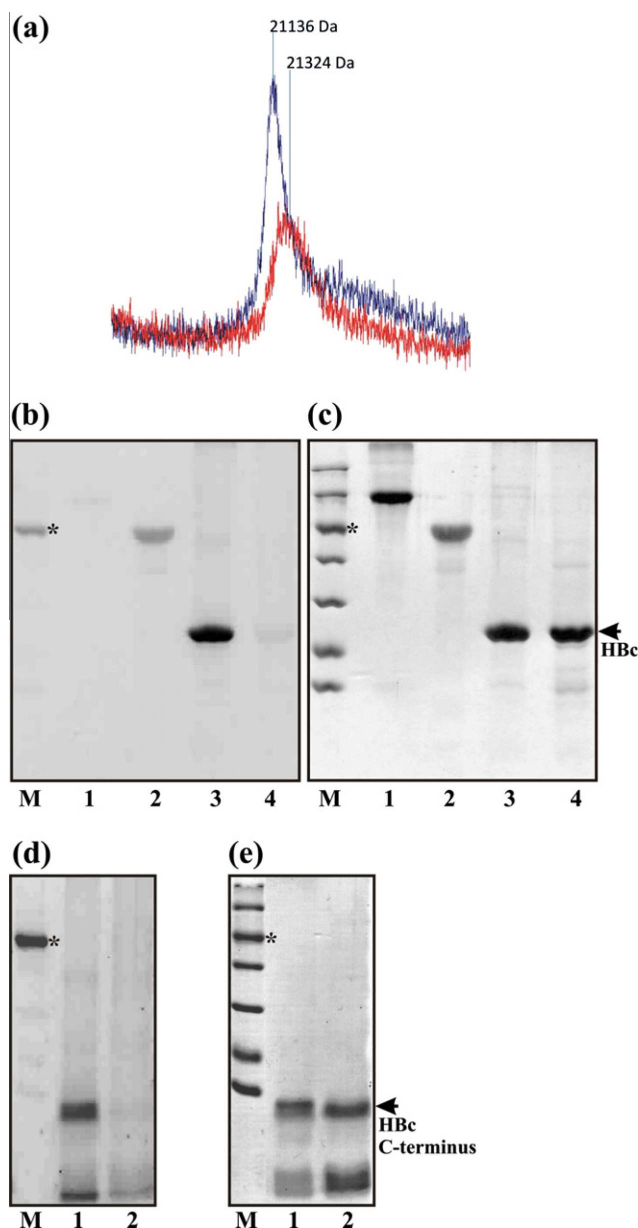

Fig. 4. Phosphorylation of *P. pastoris*-derived HBc. Comparison of full-length yeast- and *E. coli*-derived HBc molecules by mass spectrometry (a), and by denaturing SDS–PAAG stained directly with phosphoprotein stain (b), and subsequently with CBB (c). BSA and pepsin were loaded as negative and positive controls (lanes 1 and 2, respectively). Lanes 3 and 4 represent *P. pastoris*- and *E. coli*-derived HBc, respectively. Lower panel represents denaturing SDS–PAAG with proteinase K-cleaved C-terminal domains of *P. pastoris*- (lane 1) and *E. coli* (lane 2)-derived HBc stained directly with phosphoprotein stain (d), and subsequently with CBB (e). M, protein MW standards. Phosphoprotein ovalbumin is marked by an asterisk.

be somewhat heterogenous, which is a common outcome due to partial post-translational modifications (Fig. 4a).

According to Liao and Ou [27], native HBc is phosphorylated at three serine residues at the carboxy-terminal part of the molecule. In contrast, *E. coli*-derived HBc was found non-phosphorylated [28]. To investigate whether our yeast-produced protein is phosphorylated, we subjected full-length HBc molecules to SDS–PAGE and performed phosphoprotein and CBB staining (Fig. 4b and c). The results clearly indicated that the yeast-produced HBc is indeed phosphorylated. Furthermore, we attempted to localize the phosphorylation sites. First, we performed mass spectrometry of tryptic peptides obtained from in-gel digestion of both yeast- and bacteria-derived HBc. Comparing both spectra, one peptide with a MW of 1553 Da (corresponding to the sequence DLVVSYVNTNMGLK) was present only in the bacteria-derived protein, but a peptide with a MW of 1630 Da was present only in the yeast-expressed material (data not shown). Because the difference in both MWs corresponds to the MW of a phosphate group, we conclude that phosphorylation has occurred in the DLVVSYVNTNMGLK peptide, presumably on the Ser87 residue, phosphorylation of which was recently demonstrated *in vitro* [52]. No other similar differences in mass spectra could be observed. However, potential phosphorylation sites in the C-terminal part of the molecule, similar to those observed in the native virus, are surrounded by frequent arginine residues, resulting in very short tryptic peptides that are difficult to observe with mass spectrometry. Therefore, to investigate whether there are any phosphate groups added to the C-terminal part of the polypeptide, we performed a cleavage with proteinase K, which should produce a long C-terminal peptide with a MW of 4360 Da. Cleavage products were loaded onto SDS–PAAG, and staining was performed with both phosphoprotein and Coomassie stains. The results indicated that the C-terminus of yeast-derived HBc is phosphorylated, too (Fig. 4d and e). This is consistent with data from whole protein mass spectra, indicating that there might be 2–3 phosphorylation sites per monomer. In conclusion, we have determined one phosphorylation site at Ser87 and another at the C-terminal part. Although we do not have exact experimental evidence, we speculate that the phosphorylation site(s) at the C-terminus might be the same as for the native virus, at one or several of the residues Ser155, Ser162, and Ser170. Structurally, Ser87 is located in an alpha helix, forming 4-helix bundle spikes on the surface of HBc VLPs. It should be noted that a study of Watelet et al. [23] regarding *Pichia*-derived HBc did not reveal any phosphorylation sites, although phosphoprotein detection was attempted. However, HBc from the HBV *adw* subtype contains Asn87 instead of Ser87 and therefore cannot be phosphorylated. Also, different expression conditions might influence the efficiency of phosphorylation at the C-terminal domain of the HBc molecule.

Reversible natural phosphorylation of the HBc is essential for distinct steps of HBV replication, such as pregenome packaging, plus strand DNA synthesis, capsid localization, and virus maturation and secretion [53]. Since exposure of the nuclear localization signals depends on phosphorylation of the HBc, the latter emerges as a prerequisite for transport of the viral genome to the nucleus [54]. Therefore, simple and efficient production of phosphorylated HBc particles paves a way for further functional investigations of intimate HBV replication mechanisms. Although 90% purity level is not ideal, this is close to the maximum because repeated cycles of gel-filtration or AEC did not produce any improvements (data not shown). We strongly suggest that remaining impurities have internal capsid localization and could not be removed by conventional purification methods. Controlled dis- and re-assembly of obtained VLPs in combination with packaging of therapeutic substances (e.g., antibiotics, CpG oligonucleotides, and thermotherapy agents) will be a subject for further investigation.

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