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Purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris* and *in vivo* analysis of their immunogenic properties

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ABSTRACT

Following earlier studies on high-level intracellular production of hepatitis B surface antigen (HBsAg) using recombinant *Pichia pastoris*, we present here in detail an enhanced method for the purification of recombinant HBsAg virus-like particles (VLPs). We have screened various detergents for their ability to promote the solubilization of recombinant intracellular HBsAg. In addition, we have analyzed the effect of cell disruption and extraction regarding their impact on the release of HBsAg. Our results show that introduction of the mild nonionic detergent Tween 20 in the initial process of cell lysis at ~600 bars by high pressure homogenization leads to the best results. The subsequent purification steps involved polyethylene glycol precipitation of host cell contaminants, hydrophobic adsorption of HBsAg to colloidal silica followed by ion-exchange chromatography and either isopycnic density ultracentrifugation or size exclusion chromatography for the recovery of the VLPs. After final KSCN treatment and dialysis, a total yield of ~3% with a purity of >99% was reached. The pure protein was characterized by electron microscopy, showing the presence of uniform VLPs which are the pre-requisite for immunogenicity. The intramuscular co-administration of HBsAg VLPs, with either alum or a PEGylated-derivative of the toll-like receptor 2/6 agonist MALP-2, to mice resulted in the elicitation of significantly higher HBsAg-specific IgG titers as well as a stronger cellular immune response compared to mice vaccinated with a gold standard vaccine (Engerix™). These results show that *P. pastoris* derived HBsAg VLPs exhibit a high potential as a superior biosimilar vaccine against hepatitis B.

1. Introduction

The development of a safe recombinant hepatitis B vaccine has led to the inclusion of hepatitis B vaccination in the national infant immunization schedules of approximately 160 countries [1]. Recombinant DNA technology was used to produce hepatitis B surface antigen (HBsAg) in form of virus-like particles (VLPs) using the yeast *Saccharomyces cerevisiae* leading to the development of a so-called "second" generation hepatitis B vaccine and the first recombinant subunit vaccine available [2]. This formulation of the hepatitis B vaccine has been on the market since 1986. Initially, HBsAg VLPs of ~22 nm were purified from the plasma of asymptomatic HBV carriers, but due to safety issues and restricted supply, the "first" generation plasma-derived vaccines are no longer in use [2]. Nowadays, as patents have expired, "third" generation "biosimilar" recombinant HBsAg VLP-based vaccines are being introduced into the market by a variety of new manufacturers which try to make the vaccine also more affordable to developing countries [2].

As HBsAg is a very hydrophobic protein, secretion is inefficient in yeast and high-level production has been only achieved as intracellular product. The purification of recombinant HBsAg from

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Table 1
Snapshot view on purification processes for hepatitis B surface antigen from yeast cultures.

Host	Purification steps	Ref.
S. cerevisiae	Lysis → centrifugation → Amicon concentration → XAD-2 → centrifugation → Aerosil-380 → butyl agarose	[3]
S. cerevisiae	Lysis → centrifugation → Aerosil-380 → ammonium sulfate precipitation → Sepharose 4B	[4]
S. cerevisiae	Lysis → centrifugation → Aerosil-380 → ECTHAM-cellulose → Sepharose 6B → ammonium thiocynate treatment → dialysis	[5]
S. cerevisiae	Lysis → centrifugation → urea treatment → Aerosil-380 → Amicon concentration → Sepharose CL-4B → dextran sulfate → CsCl ultracentrifugation	[6]
S. cerevisiae	Lysis → PEG followed by acetic acid treatment → calcium chloride treatment → centrifugation → Amicon concentration → Fractogel TSK HW65(F) → Fractogel TSK DEAE 650 (M) → Fractogel TSK HW65(F)	[7]
S. cerevisiae	Lysis → PEG followed by acetic acid treatment → calcium chloride treatment → centrifugation → Amicon concentration → Fractogel TSK HW65(F) → Fractogel TSK DEAE 650 (M) → Fractogel TSK HW65(F)	[8]
S. cerevisiae	Lysis → centrifugation → solubilization using Triton X-100 → concentration → diafiltration → urea treatment → diafiltration → KSCN → dialysis	[9]
S. cerevisiae	Lysis → acidification → centrifugation → ammonium sulfate precipitation at pH 6.5 → centrifugation → suspension of precipitate → dialysis → hydroxyapatite (repeat: 2 times) → dialysis followed by ultrafiltration	[10]
S. cerevisiae[a]	Precipitation → immunoaffinity chromatography → size-exclusion chromatography	[11]
S. cerevisiae	Lysis → centrifugation → PEG precipitation (8%) → centrifugation → pellet suspension and homogenization → PEG precipitation (3%) → centrifugation → PEG precipitation (8%) → Centrifugation → Pellet suspension and homogenization → diafiltration → sucrose density gradient centrifugation → ultrafiltration → CsCl ultracentrifugation → diafiltration → ultrafiltration → TSK HW 65 → CsCl ultracentrifugation → dialysis and ultrafiltration	[12]
S. cerevisiae	Lysis → centrifugation → detergent treatment → centrifugation → XAD-4 → hydrophobic interaction chromatography	[13]
H. polymorpha	Lysis → precipitation of cell debris with PEG → separation of PEG supernatant → adsorbtion on a silica matrix → separation of the silica matrix → desorption of the product from the silica matrix → separation of the supernatant of the silica matrix → ion exchange chromatography → ultrafiltration → density gradient ultracentrifugation → size-exclusion chromatography → sterile filtration	[14]
H. polymorpha	Lysis → centrifugation → anion exchange chromatography → butyl-S QZT → ultrafiltration → size-exclusion chromatography	[15]
P. pastoris	Lysis → acid precipitation → Hyflo Super Cel	[16]
P. pastoris	Lysis → centrifugation → Amberlyte XAD-2 column → Macroprep High Q chromatography → cellufine sulfate chromatography → ultrafiltration → formulation	[17]
P. pastoris	Lysis → centrifugation → treatment with colloidal silica → Macroprep High Q chromatography → butyl Sepharose-4 fast flow → ultrafiltration → Sepharose CL-4B → ultrafiltration → formulation	[18]
P. pastoris	Lysis → centrifugation → acid precipitation → Aerosil-380 → immunoaffinity chromatography → ion-exchange chromatography → size-exclusion chromatography	[19]
P. pastoris	Lysis → centrifugation → Aerosil-380 → DEAE Toyopearl 650M → HiLoad Superdex 75	[20]
P. pastoris	Lysis → centrifugation → ultrafiltration of supernatant → immunoaffinity purification → ultrafiltration	[21]
P. pastoris	Lysis → centrifugation → membrane extraction with detergent → centrifugation → "HIMAX" technology → centrifugation → DEAE → diafiltration	[22]
P. pastoris	Lysis → precipitation → centrifugation → Phenyl-5PW HIC → ultracentrifugation	[23]
P. pastoris	Lysis → PEG precipitation → centrifugation → Aerosil-380 → DEAE Sepharose FF → ultracentrifugation → KSCN treatment and dialysis → formulation	[24]
P. pastoris	Lysis → centrifugation → membrane extraction → centrifugation → PEG precipitation → centrifugation → diafiltration → phenyl 600M → size exclusion chromatography → dialysis	[25]

[a] HBsAg was secreted into the culture medium.

yeast cultures is well documented [3–25] [see Tables 1 and 2] and several studies have shown that purified yeast-derived HBsAgcan assemble into characteristic ~22 nm VLPs [26–29]. These particles are highly immunogenic and capable of eliciting potent neutralizing antibodies as they mimic the conformation of native viruses but lack the viral genome and can be used as safe and cheap vaccine [26,30–32].

Previously, we have reported a simple fed-batch technique which leads to the production of ~6–7 g/l HBsAg, with 30% in a "soluble" form competent for assembly into VLPs [29]. Although, the purification of HBsAg VLPs was reported before in the Methods section [24], optimization studies of the extraction conditions, details of the purification of HBsAg VLPs and the final characterization of their immunogenic properties were not reported. Here, a simple strategy is outlined for the purification of HBsAg leading to VLPs with satisfactory yields, high purity and excellent quality. Finally, we provide evidence in mice about the superior immunogenic properties of these HBsAg VLPs as a parenteral subunit vaccine in combination with either alum or a novel adjuvant, the TLR2/6 agonist MALP-2.

Table 2
Purification of HBsAg from yeast cultures using ultracentrifugation (UC) or size exclusion chromatography (SEC) as final step (prior to KSCN treatment) [a]

Yeast	Purification steps[b]	Final recovery[c] (mg/l culture broth)	Purity[c] (%)	Reference
S. cerevisiae	6^{UC}	~0.3	90	[6]
S. cerevisiae	13^{UC}	nd	nd	[12]
P. pastoris	3^{UC}	10	nd	[23]
P. pastoris	4^{UC}	nd	nd	[24]
S. cerevisiae[d]	3^{SEC}	~0.06	nd	[11]
H. polymorpha	5^{SEC}	nd	95	[14]
H. polymorpha	4^{SEC}	nd	99	[15]
P. pastoris	4^{SEC}	nd	95	[17]
P. pastoris	5^{SEC}	nd	95	[19]
P. pastoris	$4^{UC\ or\ SEC}$	~50	>99	This study

UC (ultracentrifugation), SEC (size exclusion chromatography), nd (not determined).
[a] Only references on HBsAg purification included containing respective quantitative data.
[b] Number of purification steps before final ultracentrifugation (UC) or size exclusion chromatography (SEC); normal centrifugation step is not considered as purification step.
[c] Recovery and purity relates to the final pure bulk protein.
[d] HBsAg was secreted into the culture medium.