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Short paper

Stability of S-HBsAg in long-term stored lyophilised plant tissue

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ABSTRACT

A potent plant-derived oral vaccine against Hepatitis B Virus (HBV) requires a durable and compact form for efficacious and convenient distribution and delivery. In the previous study we have devised a successful freeze-drying process of plant material containing the HBV small surface antigen (S-HBsAg) for the purpose of an oral vaccine against the virus, but product storage stability was limited to 4 °C. The aim of this study was to upgrade a freeze-dried product formula to facilitate successful long-term storage of S-HBsAg assembled into Virus-Like Particles (VLPs) at elevated temperatures. Series of additional excipients and storage conditions were tested. Atmosphere of nitrogen proved to preserve S-HBsAg VLPs most efficiently, with only minor degradation at the highest temperature of 37 °C. As a result, a semi-product for the oral plant-derived vaccine against HBV with good storage capabilities was obtained.

1. Introduction

The alarming prevalence of Hepatitis B Virus (HBV), coupled with deficiencies in vaccination programmes stimulate research on a new type of effective, but inexpensive and commonly available vaccine. Potential plant-based anti-HBV vaccines pose an excellent tool for mass prevention as their production costs are comparable with microbial bioreactors and much better than mammalian systems and orally delivered plant-produced VLP-assembled antigens could stimulate local and systemic immune response [1]. Oral route of vaccine delivery eliminates complex purification steps and minimizes required medical facilities. Formula optimised for cold-chain free distribution would further simplify associated logistic.

Freeze-drying or lyophilisation is widely used to preserve pharmaceutical proteins, which are physicochemically unstable in aqueous solutions [2]. However, designing successful lyophilisation process does not assure long-term stability of dried formula [3]. In the past decades numerous critical factors were identified regarding protein stability during lyophilisation and long-term storage. Still, precise mechanisms of protein preservation in the solid state have not been completely understood. Therefore, development of freeze-drying protein drug formulation still remains an empirical effort to some extent.

During the previous study we have optimised freeze-drying process of plant material containing the small surface antigen (S-HBsAg) of HBV for the purpose of a potential oral vaccine against the virus [4]. Established drying conditions and sucrose as excipient proved successful in inhibiting lyophilisation-induced degradation, preserving native structure of the antigen, its Virus-Like Particle (VLP) organisation and immunogenicity. Unfortunately, VLPs were stable only at 4 °C, while at 22 °C and 37 °C gradually disintegrated during one-year storage. Here we present research on improving the long-term stability of S-HBsAg in lyophilised plant tissue using various additives and conditions based on real-time tests.

Most common destabilisation factors, as molecular mobility, oxidative and water activity and protein aggregation [5–9] were countered to select and prevent most significant S-HBsAg degradation pathways. To limit molecular mobility, glycine as bulking agent was employed, due to its wide use and additional advantages, including non-toxicity, high eutectic temperature, and easy crystallisation [10]. To alleviate residual water content and resulting activity, addition of desiccant was tested [11,12]. Oxidation, being a major degradation route for dried therapeutic proteins [7,9,13], was prevented by sealing vials under neutral atmosphere and addition of two antioxidants: sodium sulphite and ascorbic acid, which had been chosen due to their common use and compatibility with oral administration requirements. Some reports also indicated

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beneficial impact of divalent metal ions, especially Zn^{2+}, on protein preservation during freeze-drying [14,15]. Hence, its possible impact on the long-term stability of S-HBsAg was studied. Regarding protein aggregation, dominant view is becoming that the main effort should focus towards systems that rather than trying to prevent the association of proteins should avoid or counter protein alteration in the first place [13].

2. Material and methods

2.1. Plant material

The material used for lyophilisation experiments were blade parts of leaves harvested from progeny plants of previously obtained lettuce [16] expressing S-HBsAg at level ranged from 4 to 35 μg/g FW.

2.2. Freeze-drying and storage of lyophilised tissue

Fresh leaf blades were soaked with excipient(s) and freeze-dried (BETA 1-16, CHRIST®, Germany) according to previously selected temperature profile [4]. The soaking solution was composed of sucrose 500 mM (17% w/v) as a basic excipient, alone or mixed with another excipient: glycine 2 M (9% w/v), zinc sulphate ($ZnSO_4$) 6 mM and sodium sulphite (Na_2SO_3) or ascorbic acid, both 10 and 100 mM. All chemicals used were of analytical grade and purchased from Sigma, Germany. The lyophilised tissue was powdered and portioned to glass vials for storage, tightly sealed and placed at 4, 22 or 37 °C for up to 12 months. In respective variants, a desiccant (silica gel beads) and/or nitrogen atmosphere were applied. All preparations were analysed for residual moisture (RM) using the gravimetric method [17] and for S-HBsAg content.

2.3. ELISA and Western blot of S-HBsAg assays

The content of S-HBsAg was assayed in lettuce leaves and in derived lyophilised tissue using quantitative sandwich ELISA tests, separately for VLP-formed and total antigen, as previously described [4]. The S-HBsAg concentration was calculated in micrograms per gram of lyophilised dry weight (DW). Antigen preservation during storage was calculated as a ratio of S-HBsAg at a given time point to the level of the antigen content at the starting point. Each experimental variant was tested in three replications and analysed statistically (the variance for one-way classification with the Duncan test, $p \leq 0.05$) using Statistica 8™ software (StatSoft®). Antigen presence in stored lyophilised tissue was confirmed according to previously described Western blot protocol [4], but with prepared samples being stored for 48 h at 4 °C before analysis.

3. Results and discussion

During this study several powder formulations were screened with respect to the long-term stability of the S-HBsAg with its level monitored after a period of 3 and 12 months (Fig. 1). Decisive parameters for the selection of the most effective lyophilised tissue formula and storage conditions were efficient retention of VLPs together with a minimal change in the total antigen pool associated with release of antigen dimers from VLPs and/or their denaturation, as described earlier [4].

Additives showed no effect on the freeze-drying process efficiency itself, as S-HBsAg preservation was over 80% for VLPs and

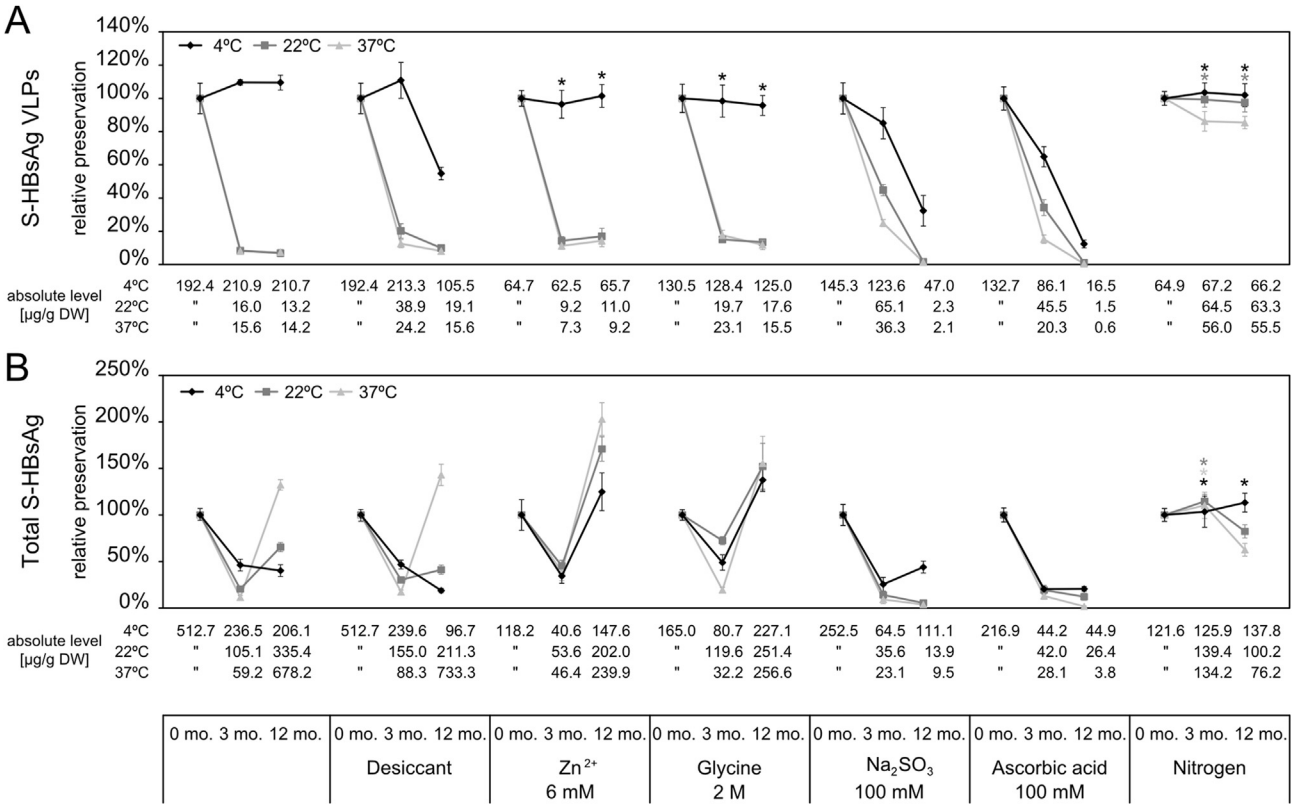

Fig. 1. Stability of VLPs (A) and total (B) S-HBsAg in freeze-dried tissue stored for 1 year at 4 °C, 22 °C and 37 °C. Material was infiltrated with 500 mM sucrose supplemented with different additives and stored under different conditions (with/no desiccator, N_2 atmosphere). Preservation efficiency is represented as relative change of antigen level to the storage start point. Star indexes mark statistically homogenous groups in regard to respective starting point (unchanged antigen content), separately for VLP-assembled S-HBsAg and the total antigen. For detailed statistical analysis see Supplementary Table.