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# Thermostability of Freeze-Dried Plant-Made VLP-Based Vaccines

Marcin Czyż and Tomasz Pniewski

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## Abstract

Freeze-drying or lyophilisation is a technique commonly used for pharmaceutical products, in which stability is required to be preserved beyond 4°C temperature. Although lyophilisation is a well-established method, designing a sustainable process for a new product is still to a large extent subjected to empirical practice and often requires trial and error approach. Moreover, even successful lyophilisation of the product may not assure its good long-term storage stability, and progressive decrease in activity may still be encountered. In the past decades, numerous studies have been conducted in the area of protein instability and preservation during lyophilisation and long-term storage. Many critical issues have been identified with regard to physical and chemical instability of proteins in the solid.

Our research effort was focused on designing the prototype oral plant-made vaccine against hepatitis B virus (HBV), which is based on expressing viral surface antigens: small, medium and large (S-, M- and L-HBsAg), assembled into virus-like particles (VLPs) in lettuce plants. We describe in detail the process of developing a freeze-drying protocol facilitating successful processing of plant tissue containing particular HBsAg antigens, while preserving their VLPs structure and immunogenicity. The processing of all HBsAg proteins was investigated, yet the research was focused on S-HBsAg as the basic structural antigen and the main viral immunogen, applied in commonly used preventive anti-HBV vaccines. Each lyophilisation step was consecutively evaluated. Protective excipients, freeze-drying profile, associated processes and long-term storage were investigated to select the most effective conditions. Finally, sucrose demonstrated appropriate properties as a lyoprotectant and the profile of 20°C for 20 h for primary and 22°C for 2 h for secondary drying, as well as nitrogen atmosphere for long-term storage, enabled to maintain S-HBsAg VLP structure and antigenicity.

The preservation of native and immunogenic S-HBsAg in plant-derived preparation was confirmed during mouse immunisation trials when the orally administered preparation was used as boosting and elicited an immune response comparable to

routine injection vaccination. Obtained results provided basis for an efficient freeze-drying process that in the future can be utilised for the purpose of a suitable oral plant-derived vaccine against HBV. On the example of anti-HBV oral vaccine, this chapter presents comprehensive view on the development process and main problems, which can be faced during work on freeze-dried products containing bioactive proteins of plant origin. A significant insight into practical aspect of designing successful parameters for material that is not widely used, but gains attention, was given.

**Keywords:** Freeze-drying, excipient, long-term storage, neutral atmosphere, plant-made oral vaccines, plant tissue, thermostability, HBsAg, VLPs

## 1. Introduction

The original concept of plant oral vaccines dates back over 20 years. Some of the first and flagship research projects were studies on vaccines against hepatitis B virus (HBV).

To date, the epidemiological situation connected with HBV around the world, particularly in developing countries, has not improved markedly [1, 2]. The first attempts at providing a solution to this problem by oral vaccination were based on the idea of immunisation through consumption of raw tissue of transgenic plants expressing HBV surface antigens (HBsAg). However, despite some encouraging results [3–8], as induction of a specific humoral response [9], it was soon outlined that the future oral vaccine cannot be founded on unprocessed raw plant material [10, 11]. A practical application of fresh plant material in the form of a medication would present a number of problems in both the distribution and the application itself. Limited durability, uneven antigen content throughout the harvested biomass and fostering oral tolerance acquisition were only some of the major forecasted problems. Therefore, the original concept evolved from plant-based ‘edible’ vaccine to orally delivered lyophilised preparation.

However, freeze-dried formulations facilitate elimination of complex material purification, size reduction and better stability during storage, as well as easy handling and a controlled administration regime, including dosage and short-duration delivery. This was highly attractive with regard to priorities of efficacious, cost-effective, and reliable mass hepatitis B vaccination programmes in developing countries [1, 2, 12, 13]. Preliminary trials confirmed that freeze-dried material containing the small surface antigen of HBV (S-HBsAg) without exogenous adjuvants induced a systemic immune response above the nominal protective titre in mice. Nevertheless, lyophilisation of plant material required further investigation, since 90% degradation of S-HBsAg and other surface antigens—medium and large (M- and L-HBsAg) in the specific immunogenic form assembled into virus-like particles (VLPs)—was observed during that process [10, 11].

## 2. Basics of protein lyophilisation

Freeze-drying or lyophilisation is widely used to preserve biologically active proteins and polypeptides, including biopharmaceuticals, which are physically and/or chemically unstable

in aqueous solutions [14]. While it is a well-established method, commonly used for pharmaceuticals, it continues to be subjected to empirical practice in terms of its optimisation or adaptation to new candidates [15, 16]. This method has great potential for improving the stability of labile substances, especially proteins [17] and liposomes [18, 19], and also generates physicochemical stresses, which can denature proteins to various degrees. In addition, even after successful lyophilisation, the obtained product may still have a limited long-term storage stability. To preserve protein from denaturation caused by freezing (cryoprotection) and/or dehydration (lyoprotection), a stabilising excipient(s) may be used, in parallel to establishing optimal process profile [15, 17, 20].

Freeze-drying is a complex process involving several steps and depending on various factors. Each of them can have a direct or indirect impact on the efficiency of the process, which is defined as preservation of a protein in the biologically active form. In order to achieve high efficacy, both lyophilisation parameters and material formulation must therefore be carefully optimised. In general, a freeze-drying process comprises three stages, namely freezing (material solidification), primary drying (ice sublimation) and secondary drying (moisture desorption). Efficiency of a freeze-drying cycle depends on process variables, such as cooling rate, shelf temperature and duration, combined with the presence of protective additives [16, 17, 20].

A variety of substances are used as effective formulation excipients for their cryoprotective and/or lyoprotective qualities, among which sugars and polyols are the most common [16, 17]. These can serve both as the amorphous phase protecting an active agent and as a bulking component providing desired physical properties of a lyophilised solid, sometimes expressing those abilities in parallel.

Mechanisms of protein preservation in the dry state are still not sufficiently understood to explain and predict the stability after a protein loses its hydration shell [21, 22]. There are two main hypotheses that provide some explanations of protein stabilisation during lyophilisation and subsequent storage in a dehydrated state. The first is the water substitute hypothesis [23–25], and the other is the glass dynamics hypothesis [26]. However, neither sufficiently explains all the observed aspects of freeze-drying.

The water substitute hypothesis assumes that physical denaturation of a protein is inhibited according to the principles of thermodynamics. Preservation of hydrogen bonds maintains a low value of free energy and thus protects the native protein structure. During drying, substances, such as sucrose or polyols, can form hydrogen bonds by their hydroxyl groups at specific locations on the surface of a protein, substituting removed water and stabilising a protein [17, 27]. Thermodynamic stabilisation is ensured by maintaining balance between native conformation and the unfolded structure. A stabiliser increases the free energy of unfolding of a protein and thereby shifts the equilibrium towards its native, more stable state.

The glass dynamics hypothesis assumes that a stabilising agent forms a glassy matrix with very high viscosity, which extremely reduces molecular mobility. This glass is formed by substances, such as sucrose or mannitol, which do not or only partially crystallise during the solution cooling process. The point at which a dissolved substance (liquid state) passes into a

glass with a very high viscosity is referred to as glass transition temperature ( $T_g$ ). However, this conversion is not considered as a physical phase transition [28]. In contrast to crystallisation, the glassy state preserves an interaction between the excipient and the protein, thus allowing limited mobility to be extended to a protein and consequently significantly slowing degradation processes. However, at temperatures above  $T_g$ , the system may enter into a more mobile state. If a specimen is maintained in this state for a sufficient time, irreversible degradation may occur especially in the case of complex structures such as proteins. Therefore, glass transition temperature is also referred as collapse temperature ( $T_g$ ) and during freeze-drying the temperature of a given formulation must be kept below its specific  $T_g$ . Selected excipients may increase collapse transition temperature and thereby enhance stabilisation of a formulation during both freeze-drying and storage [29].

In the first stage of the freeze-drying process, a material must be frozen, which facilitates immobilisation of solution components and prevents foaming during the vacuum stage [30]. It also facilitates the formation of a structure of ice crystals in the frozen mass, which will directly determine the rate of subsequent sublimation and the final morphology of a dried product.

Water crystallisation does not occur at the same time throughout its entire volume but progresses from nucleation sites and forms ice crystals. Since water crystallises in the form of pure ice crystals, it causes condensation of the remaining solution. Also, at this stage, an interaction forms between the lyophilised protein and protective excipients. A glassy state is created by the immobilised protein with established direct hydrogen bonds. Also, freezing has a direct impact on drying rate. Creation of large ice crystals, and thus, a highly porous product facilitates easy migration of water vapour from the product and is achieved by relatively slow freezing (0.2–1.0°C/min). Fast freezing produces small ice crystals, hindering the release of vapour from ice and thus limiting heat loss, which leads to overheating and product collapse [21, 31, 32]. However, slow cooling promotes inactivation of dried proteins due to their prolonged exposure to high local concentrations of different chemicals, pH shift, phase separation or cell membrane dehydration as liquid water is transformed into ice crystals [33–35].

With the progress of cooling, the formed material reaches the point, at which its entire volume is solidified. Typical lyophilised formulations are not subject to crystallisation of all components during freezing. Active proteins and added protective substances remain in a non-crystalline, glassy form, which allows them to interact.

Annealing is an optional step often included after freezing and before drying. It is performed by raising the temperature of a formulation after it is completely frozen and maintaining it for a specified period [33]. This step is used for the recrystallisation of all the components that were not completely solidified during freezing. Leaving a pool of ingredients in the glassy state may lead to their spontaneous crystallisation during drying or subsequent storage, which in turn may result in the release of free energy and hence plasticise the glassy phase comprising the excipient and processed protein. However, sometimes the annealing process may also lead to crystallisation of the amorphous protective component, which will result in the elimination of interactions with stabilised protein and its subsequent inactivation [17]. Annealing is also used to convert the ice structure into large crystals, which improves the sublimation rate,



resistance to temperature and ensures a more uniform crystalline structure of the entire dried batch.

Drying, the next step of lyophilisation, consists of two stages: primary and secondary drying. The primary stage begins with lowering the pressure in a lyophiliser chamber below the limit of the water triple point, which allows sublimation to start. The optimum pressure range in the chamber is 0.15–0.2 mbar, while fluctuations in this range have little effect on drying efficiency [36, 37]. Lowering pressure below a certain point may even decrease sublimation rate, as high vacuum will isolate the product from the heated shelf and limit heat transfer needed for evaporation [14]. A significant increase in the sublimation rate and shortening of the process duration are achieved almost exclusively by increasing sample temperature. Hence, optimisation of process conditions at this stage focuses mostly on determining a maximally high shelf temperature, but avoiding melting or collapse of a product, leading to the loss of its activity. The end of primary drying is indicated by an asymptotic increase in product temperature up to the temperature of the lyophiliser shelf [38]. At this point, all crystallised ice is removed, and the remaining water content of about 5–30% in the product is in the form of the hydration shell or water structurally bound to a polypeptide [39].

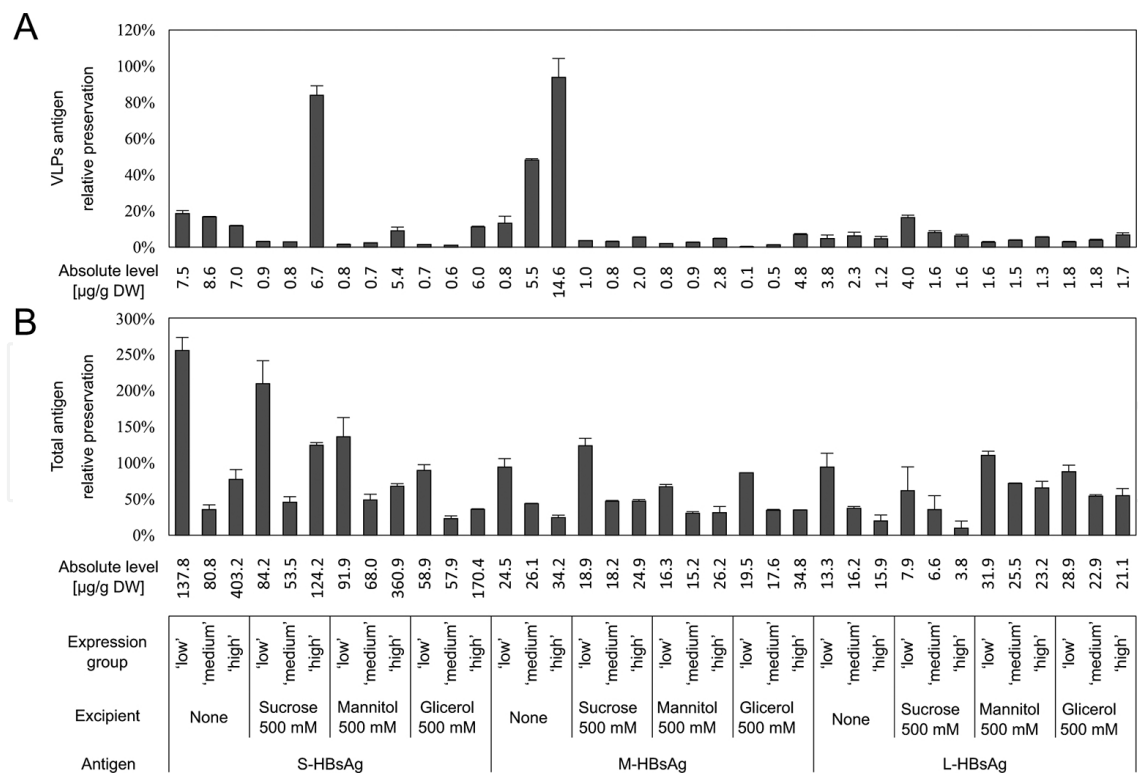
In contrast to primary drying with a high-intensity vapour flow, the secondary stage is much less effective, removing about 5–10% of total moisture in a sample, but it takes 20–40% time of the whole process. At this step, remaining moisture is removed in response to an elevated shelf temperature and application of intense vacuum [20]. Although the chance of collapse or degradation of a sample is much lower than that for primary drying, it is still possible. Moreover, complex formulations, such as proteins, often require a certain level of residual moisture to ensure good stabilisation of their structure (even up to 5–7%), but in some cases, a very low water content (max. 0.1%) is needed. Therefore, the length of secondary drying is determined by the nature of the product itself [40, 41].

### **3. Initial lyophilisation trials of plant tissue containing S-, M- and L-HBsAg**

The first stage of studies on a plant-derived oral vaccine against HBV was to investigate the stability of viral surface antigens: S-, M- and L-HBsAg contained in lettuce leaves during lyophilisation process. The surface proteins are encoded by a common gene with three autonomous start codons within the same reading frame. Therefore, all proteins contain the common and the largest domain S, which alone consists of S-HBsAg, and carries additional preS domains at its N-terminus: preS2 in the case of M-HBsAg, while L-HBsAg has preS2 and preS1. The S domain/protein once synthesised, dimerise by hydrophobic and disulphide bonds. Dimers are basal structural units of the HBV envelope and they also can self-assemble into VLPs, both naturally and in artificial expression systems. Hydrophilic preS domains are orientated to the outside of the virions or natural VLPs, consisted of S- and other HBs antigens in various proportions. Naturally, M- and L-HBsAg solely do not self-assemble into VLPs, although such recombinant VLPs can be obtained to some extent.

The plant material for lyophilisation was harvested from transgenic plants stably expressing individual HBV antigens and divided into three groups on the basis of their expression level: ‘low’, ‘medium’ and ‘high’. This categorisation aimed to determine the effect of the initial antigen content, including the behaviour of VLP-formed antigens, on process efficiency. Since the accumulation of particular antigens varied significantly, respective expression groups were different. In the case of S-HBsAg, the ‘low’ group was <2 µg of the antigen per g fresh weight (FW), ‘medium’ 2–10 µg/g FW and ‘high’ >10 µg/g FW, respectively, and for M-HBsAg, the cut-off points were <2, 2–4 and >4 µg/g FW and for L-HBsAg: <1.5, 1.5–3 and >3 µg/g FW, respectively.

Pilot variants comprised untreated material, directly harvested from plants and plant tissue soaked with three excipients, such as sucrose, mannitol and glycerol, at a concentration of 500 mM. Sucrose was selected due to its high efficiency and extensive use in freeze-drying, mannitol as an agent with the potential to form crystals as a rigid structure supporting the glassy phase formed by plant tissue proteins, and glycerol due to its low molar mass and a small molecule size, which could promote its tighter interaction with the tested antigen [13, 16–18, 20, 22, 42, 43]. Freeze-drying was performed at shelf temperatures of 20°C for 20 h for primary drying and 22°C for two hours for secondary drying (profile of 20°C/20h–22°C/2h). Obtained formulations had the desired dry powder form, with the exception when glycerol was used. In that case preparations showed signs of increased viscosity and the tendency to



**Figure 1.** Relative lyophilisation efficiency [%] of lettuce leaf tissue dependent on diverse contents of S-, M- and L-HBsAg in relation to VLPs (A) and total antigen (B). Plant expression groups—‘low’, ‘medium’ and ‘high’ [µg/g FW], respectively, for: S-HBsAg: <2, 2–10, >10; M-HBsAg: <2, 2–4, >4; L-HBsAg: <1.5, 1.5–3 and >3.

clump. The probable reason was the high glycerol content in the tissue after drying, together with its liquid physical state at the tested temperature range of 4–37°C.

Results for individual antigens varied greatly, confirming their different sensitivity to physicochemical factors during freeze-drying. Most likely, despite having a common structural S domain, additional N-terminal preS2 and preS1 domains substantially affect sensitivity of S-, M- and L-HBsAg under stress conditions or their interactions with various excipients. Initial tests have shown low lyophilisation efficiency in most variants—around 10% of preserved VLPs (**Figure 1**), while only exceptionally it was around or more than 20%. However, when the total antigen pool was analysed, a much better efficiency was observed, amounting to 50–100%, which corresponded to several tens to several hundred µg/g dry weight (DW). However, in some samples, a substantial (apparent) increase in the total antigen level was observed. It exceeded 100% when compared to the theoretical full preservation of the antigen after drying.

According to the adopted hypothesis, the increase in the total pool of antigen is associated with the degradation of VLPs formed by HBs antigens. Plant cells express antigens in two pools: one comprises properly assembled VLPs, while the other is composed of antigens forming aggregates and incomplete subparticles and even unstructured ‘free’ dimers [10]. Effects of various stresses can cause fragmentation of particles, subparticles and aggregates of antigens to dimers, artificially adding to the total amount of antigens. These various forms are in fact indistinguishable in immunoenzyme assays, including commercial tests, utilising monoclonal and polyclonal antibodies specific to the S domain—common for all HBsAg. To some extent, these tests detect ‘subparticles’, which is reflected as an apparent increase in VLPs contents, also observed by other authors [13, 44]. However, further disintegration of the VLPs is no longer observed as their increase; instead, the total antigen content begins to rise. In addition, only small part of VLPs may be decomposed resulting in a large number of smaller aggregates or dimers, substantially increasing the pool of the ‘free’ antigen compared to the diminished or even constant quantity of VLPs. At the same time, it must be noted that larger the particle, the higher its susceptibility to decay due to the relatively weaker nature of the constructing bonds. The intensity or nature of a given stress causing VLPs fragmentation may be usually insufficient to denature dimers, which are stabilised by strong hydrophobic interactions and disulphide bonds. However, other stresses can cause degradation of dimers or polypeptide chains themselves, finally reducing the pool of ‘free’ antigens. Both types of degradation can occur at different times or simultaneously, with the same or different intensity. All these processes are manifested as large fluctuations of VLPs and/or total antigen levels.

Considering that the main functional component of existing and developed vaccines against HBV are VLP-formed by HBs antigens, mostly S-HBsAg, [45], optimisation of the lyophilisation process should focus primarily on the preservation of these structures. However, the level of the ‘free’ antigen should also show a minimal fluctuation, especially avoiding its growth, due to the possible side effects such as induction of oral tolerance. A small reduction of total antigen content may be acceptable due to the elimination of the unfolded antigen pool while maintaining the VLPs. In the best scenario, after freeze-drying, full preservation of VLPs and an unchanged total antigen level should be observed. However, in view of the higher stability



of dimers in comparison to VLPs, a moderate degradation of VLPs with a slight increase in the total antigen level may be reasonably expected [45].

Regarding the potential impact of an initial antigen content on lyophilisation efficiency, HBs antigens showed different effects. Processing of the material with S-HBsAg and soaked with excipients expressed a clear positive trend towards a greater antigen stability together with a higher antigen content. However, differences between the best and the worst results amounted to 81, 8 and 10%, for sucrose, mannitol and glycerol, respectively. An opposite trend was observed for untreated plant tissue, yet dispersion of efficiency was only 6%. The use of sucrose provided good antigen stabilisation in the form of VLPs amounting to 84%, which corresponded to 30.7  $\mu\text{g/g}$  DW. Mannitol and glycerol expressed a significantly lesser protective effect towards VLPs at only 9 and 11%, yet it was still higher in comparison to the variant without any excipients added. Interestingly, soaking of plant material with any excipient from the 'low' and 'medium' groups resulted in a very low freeze-drying efficiency—from 1.1 to –3.1%, even lower than that of the untreated material, indicating a varied effect of nominally protective substances depending on the type of source material. Preservation of the total antigen pool was much more erratic; however, it showed to be less susceptible to degradation than in the case of VLPs. The use of tissue from the 'low' expression group resulted in the highest total antigen contents for all variants of tissue treatment, whereas for the 'medium' and 'high' groups stabilisation was generally well below 100%. Only when sucrose was used, the total antigen pool increased moderately to 124%, in parallel to the high degree of VLPs conservation.

Freeze-drying of M-HBsAg also demonstrated a positive correlation between VLPs stability and the amount of antigen in the source plant tissue, yet the total antigen and the characteristic preS2 domain were preserved in the reverse pattern [11]. However, unlike S-HBsAg, VLPs preservation in variants with excipients was significantly lower (by up to 7%) than that for untreated tissue (>90% or 14.6  $\mu\text{g/g}$  DW). Yet again, preservation of the total antigen pool showed a reverse trend. An important fact was that freeze-drying of material from the 'low' group resulted in the least altered antigen level.

Preservation of L-HBsAg during freeze-drying showed a distinct pattern in comparison to other HBs antigens. VLPs were very unstable and their preservation in all of the tested variants oscillated around only 10%. The maximum value, 17%—corresponding to 4.0  $\mu\text{g/g}$  DW, was achieved for plant material with a 'low' antigen content, unlike other HBs antigens. The use of sucrose positively affected preservation of this antigen, similarly to S-HBsAg, but distinctly in comparison to M-HBsAg, which is structurally more related. Preservation of the total L-HBsAg and its characteristic preS1 domain was also markedly lower than other HBs antigens, decreasing gradually with an increasing initial antigen content in the plant material [11]. The obtained efficiency ranged from 62–110%, reaching a maximum 16  $\mu\text{g/g}$  DW.

Summarising this stage of research, it was found that some protective substances can increase stability of HBs antigens. Among the tested excipients, a positive effect was observed for sucrose towards S- and L-HBsAg, while mannitol and glycerol showed no significant effect on VLPs stability. The reason for this phenomenon is unknown, but a significant impact of sucrose on freeze-drying efficiency has been reported [46, 47]. Preservation of the total antigen pool

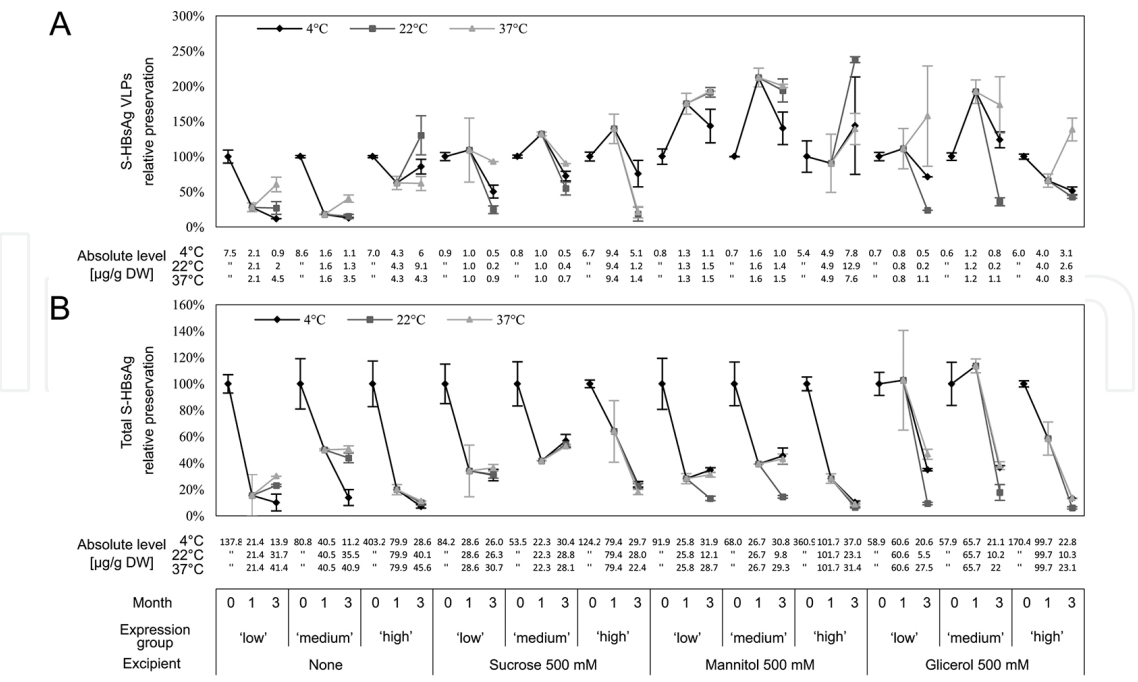
using excipients showed significantly larger (30–100%) fluctuations than for VLPs, with no visible advantage of a specific excipient. However, S-HBsAg expressed a similar behaviour of VLPs and total antigen, whereas results for the M and L-HBsAg were much more ambiguous, yet usually a higher VLPs stability was accompanied by a decreasing preservation of total antigen.

Initial tests revealed a distinct stability of VLPs, 'free' dimers and particular antigen domains, depending on the antigen structure and its initial content in plant cells, as well as the physicochemical effect of added protective substances. Observed differences could arise from different requirements for the residual water content after drying or other effects of interactions between molecules of the protective agent and VLPs surface, yet the nature of these processes remains unknown. On the basis of obtained results, it may only be assumed that S-HBsAg retained its VLPs structure due to the interactions with additional substances, most likely by creating hydrogen bonds on the surface of the particle. In turn, the antigen M-HBsAg, due to the presence of the preS2 domain located on the surface of the particles may express a self-stabilising effect and perform the role of a protective excipient in the formation of polar bonds (hydrogen, electrostatic and others) with the surface of the particle. In contrast, L-HBsAg having the largest additional polypeptide chain on the surface of the particle, composed of the preS2 and preS1 domains, may lose the ability to self-stabilise due to the influence of preS1 on the preS2 domain. In addition, a large total size of the two domains may also block access of excipients to the surface of the S domain, which during lyophilisation and water deficit may in effect weaken interactions between the S domains and contribute to the decay of VLPs. A similar effect of blocking the particle by outer domains may be responsible for the low stability of M-HBsAg VLPs in the presence of sucrose. The preS2 domains by binding excipient molecules lose their self-stabilisation ability and additionally isolate the S domain from the excipient. Somehow correspondingly to this hypothesis, in the case of lyophilisation of total antigen and the preS domains, the dominant tendency was connected with their decreasing preservation, reversely correlated with their increasing content in the source material. This phenomenon could be attributed to the limited amount of the 'free' antigen, which can be stabilised by the excipient or residual water, and/or innate properties of a particular HBsAg.

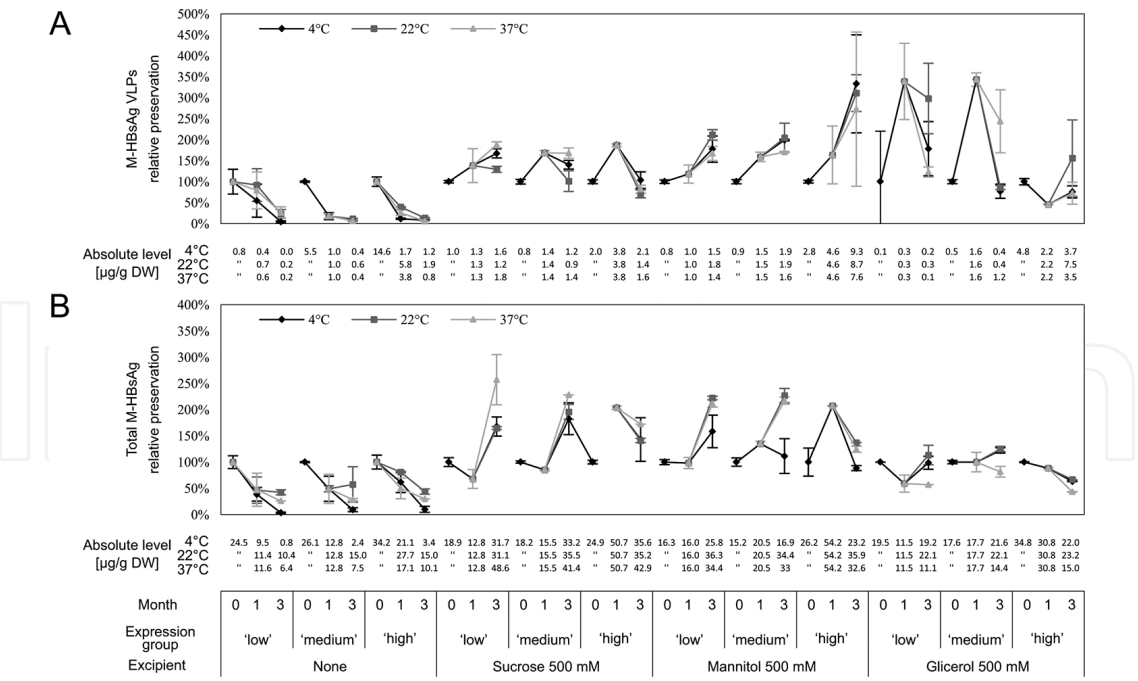
Obtained results implied that further work on the optimisation of the lyophilisation process, as well as efforts to increase stability of HBsAg antigens must focus on preserving VLPs as their more sensitive form, which constitute also the main vaccine immunogen.

#### **4. Preliminary studies on storage stability of freeze-dried S-, M- and L-HBsAg**

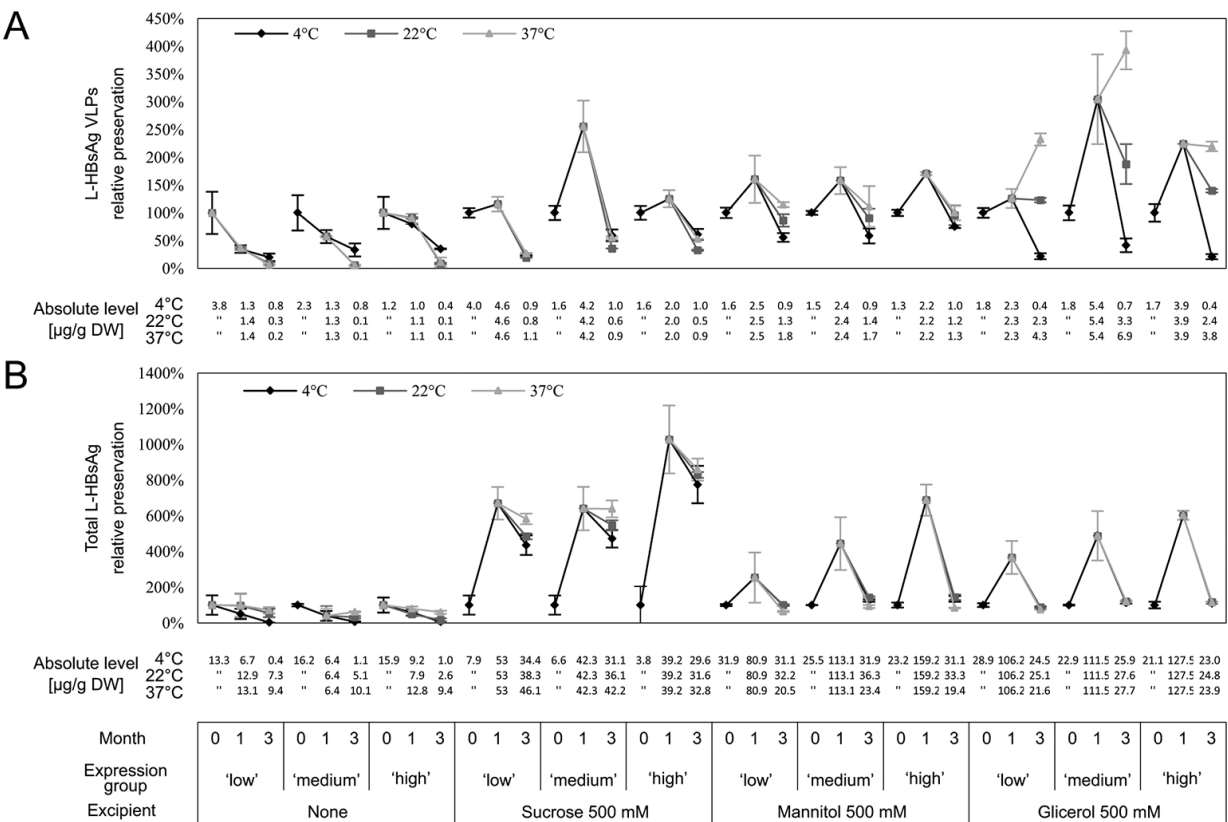
Preparations of freeze-dried plant tissue-containing S-, M- and L-HBsAg, obtained during the previous step, were subsequently analysed for the stability of the antigens during 3-month storage at temperatures of 4, 22 and 37°C. All variants showed significant fluctuations in terms of VLPs and total antigen stability (**Figures 2–4**).



**Figure 2.** Relative changes [%] of S-HBsAg VLPs (A) and total antigen (B) in lyophilisates derived from tissues of diverse antigen expression levels, then soaked with protective substances and stored at different temperatures. Antigen expression groups, respectively 'low', 'medium' and 'high': <2, 2–10 and >10  $\mu\text{g/g FW}$ .



**Figure 3.** Relative changes [%] of M-HBsAg VLPs (A) and total antigen (B) in lyophilisates derived from tissues of diverse antigen expression levels, then soaked with protective substances and stored at different temperatures. Antigen expression groups, respectively 'low', 'medium' and 'high': <2, 2–4 and >4  $\mu\text{g/g FW}$ . Total M-HBsAg assayed using the ELISA test specific to the preS2 domain.



**Figure 4.** Relative changes [%] of L-HBsAg VLPs (A) and total antigen (B) in lyophilisates derived from tissues of diverse antigen expression levels, then soaked with protective substances and stored at different temperatures. Antigen expression groups, respectively 'low', 'medium' and 'high': <1.5, 1.5–3 and >3 µg/g FW. Total L-HBsAg assayed using the ELISA test specific to the preS1 domain.

During storage of freeze-dried material containing S-HBsAg, in most variants, large fluctuations of VLPs contents were observed, with both a significant decline and an apparent increase (Figure 2A), especially at the intermediate point of the period. This should be considered as a negative phenomenon—an indication of VLPs disintegration into smaller 'subparticles' or aggregates. In this context, the smallest fluctuations of the VLPs level were observed in the formulation derived from the material with a 'high' initial S-HBsAg content, soaked with sucrose and stored at 4°C. At higher temperatures, S-HBsAg VLPs were significantly less stable. Total antigen, comprising particles and the 'free' antigen, released during their gradual disintegration, expressed heavy degradation at all temperatures, especially greater than 4°C (Figure 2B). However, again, the relatively most stable storage variant of total antigen employed sucrose as the protective agent. In untreated or mannitol-soaked tissue, the total antigen level gradually decreased. Samples treated with glycerol initially exhibited a decreased rate of antigen degradation, but later the antigen level dropped by more than 60%.

In the case of M- and L-HBsAg (Figures 3 and 4), degradation processes were more intensive than that for S-HBsAg, especially regarding L-HBsAg. The apparent increase in the particle contents of both antigens after the first month was significantly greater than for S-HBsAg, clearly indicating a strong VLPs decomposition. Material containing M-HBsAg showed large fluctuations in VLPs contents (Figure 3A), both at an intermediate storage point and at its end,



suggesting an intense VLPs disintegration and following 'free' antigen degradation. The smallest relative changes in the antigen content were observed when sucrose served as the protective excipient, with minor differences between products coming from different plant expression groups. Variants employing mannitol or glycerol underwent a significant apparent increase in M-HBsAg VLPs levels, with the exception of a relatively stable formulation obtained from plant tissue with a 'high' antigen concentration and soaked with glycerol. In addition, avoidance of any excipient for the freeze-drying process, resulted in a gradual, but extensive VLPs degradation during storage. Similar results were observed for the total antigen pool (**Figure 3B**). With the exception of formulations prepared with the use of glycerol, substantial degradation or apparent increase in the antigen level was observed, which indirectly confirmed severe degradation of VLPs and the occurrence of various stresses acting on the unstructured antigen.

Lyophilised plant tissue containing L-HBsAg showed a significant decline of the VLPs level, when no excipient was used (**Figure 4A**). Preparations containing a protective agent showed a significant apparent increase in antigen particle levels after the first month of storage with their subsequent severe reduction in the second storage period. The final VLPs content was comparable to the initial level only in formulations containing mannitol; however, it was after an apparent high increase at the intermediate stage. The level of total antigen (**Figure 4B**) in the formulations with no excipient gradually degraded until its almost complete degradation was reached. In contrast, a significant apparent increase—up to 600% of the initial value, was observed in the formulations containing sucrose. Formulations with mannitol or glycerol showed a significant increase of total antigen after 1-month storage, followed by a decrease to a value close to the initial. This clearly indicated disintegration of VLPs in the initial period, and subsequently, small aggregates and dimers were formed. Variants untreated with protective substances showed only progressive degradation of the 'free' antigen during storage. Nevertheless, at this stage of the study, it could be concluded that excipients had a positive effect on the stability of freeze-dried M- and L-HBsAg, when compared to a significant decrease in their contents in untreated tissue.

Results of preliminary storage experiments indicated a definite necessity to optimise the freeze-drying process as essential for the stability of HBs antigens in derived formulations. Although all antigens, both VLPs and 'free' forms, were gradually degraded, a clear beneficial effect of used protective excipients for their stability was observed (**Figures 2–4**). Additionally, preliminary results showed some important regularities (type of source material, excipients) and phenomena (durability of individual antigens and preS domains, VLPs disintegration), which helped to define directions for the next steps. Among the antigens, S-HBsAg showed the highest stability. This antigen is formed solely by the S domain, which is common, and the main structural element of M- and L-HBsAg antigens, even though the structure of those is heavily influenced by the preS2 and preS1 domains. Thus, examination and optimisation of freeze-drying and storage conditions for S-HBsAg could provide a basis for analogous studies on the other antigens. These facts along with the fundamental role of S-HBsAg for current and potential oral anti-HBV vaccines provided a justification for focusing our research mainly on this antigen.



## 5. Stability of S-HBsAg during plant tissue freezing

An essential step during the lyophilisation process is connected with material solidification by freezing. This often results in a strong degradation of processed proteins [48] or liposomes [49], thus has to be optimised to avoid an unwanted decrease in the contents of active components. The most common approach to alleviate freezing damage is to control the freezing rate of a product. Both fast and slow freezing rates were reported to have the potential of decreasing protein recovery following specific stresses of cryoconcentration, phase separation and cold denaturation characteristic of slow freezing and the formation of a large ice-aqueous interface during rapid cooling [50].

Limited cryoconcentration and phase separation are achieved mainly by optimising product composition and avoiding content reduction for certain substances such as NaCl or PVP. Susceptibility to cold denaturation is an innate trait of a given protein, but certain additives, for example, sucrose, can improve its resistance to damage. Degradation caused by the large ice interface may be prevented mainly by avoiding fast freezing or, if it is unfavourable, by directed ice seeding. This approach, by inducing ice formation, at higher temperatures can effectively slow down the ice growth process and as a result generates a smaller number of larger ice crystals. In the plant tissue, nucleation of ice is observed at temperatures ranging from 0 to -12°C depending on the species, tissue type and its physiological state [51, 52]. However, the ability and effectiveness of modifying this process in plant tissue are very limited and the methods to achieve this are still far from being applied on a regular basis [53]. In addition, inducing ice nucleation outside tissue cannot be effective, due to the membrane and cell wall barrier for ice propagation. Addition of bacteria or chemicals can promote nucleation inside tissue, but this is in contradiction with the principles applied for pharmaceutical formulations and would be difficult to standardise and scale [54].

For these reasons, none of the described methods could be used in the course of the presented work. It was only possible to analyse the effect of the freezing rate on S-HBsAg stability. The research was initiated by examining the effect of plant tissue freezing rate on the integrity of S-HBsAg, both VLPs and the total antigen pool. For this purpose, plant tissue underwent two standard freezing protocols, widely used on both the laboratory and the industrial scales: the 'fast' method utilising liquid nitrogen and 'slow' with a cooling rate of approximately 2°C/min performed in a cooling chamber. Frozen samples were also subjected to 'slow' and 'fast' thawing (water or air bath) to determine its possible impact on the antigen.

In both variants, a significant decrease in S-HBsAg VLPs together with an apparent increase in total antigen contents was observed (**Table 1**). A greater VLPs pool was maintained when the 'slow' freezing protocol was employed: 36–40%, as compared to 16–19% for 'fast' freezing. At the same time, a lower apparent increase in the total antigen was also observed during 'slow' freezing (12 and 21% when compared to approximately 34–36%). This was most probably associated with a lesser extent of VLPs collapse, while the formation of a large area of the ice-aqueous interface during rapid freezing was probably the main cause for VLPs decay. Due to the very high complexity of plant tissue as a processed material, VLPs could also be damaged by ice crystals. This supposition can attest to the fact that the slow cooling

protocol, which extended the impact of thickening substances on the antigen, provided a less degrading effect despite the large amounts and variety of compounds in plant tissue. The impact of thawing rate, however, was much smaller—differences between ‘fast’ and ‘slow’ thawing amounted to approximately 4% for VLPs and up to 9% for the total antigen pool. Still, for both freezing methods, subjecting the material to ‘slow’ thawing resulted in a greater stability of VLPs and a less apparent increase of ‘free’ antigen levels. Since the main functional component of anti-HBV vaccines is the VLP-formed antigen, and a greater elevation of ‘total’ antigen may cause undesirable side effects such as oral tolerance acquisition, ‘slow’ freezing and thawing were selected for future work.

Tissue treatment		S-HBsAg			
Freeze	Thaw	VLPs		Total	
		Content [µg/g FW]	Change [%]	Content [µg/g FW]	Change [%]
‘fast’	‘fast’	3.4 ± 1.1	19.2 ± 6.3	39.5 ± 1.5	134.1 ± 5.2
‘fast’	‘slow’	2.9 ± 1.1	16.5 ± 5.9	40.0 ± 1.2	135.8 ± 4.0
‘slow’	‘fast’	7.0 ± 1.4	39.5 ± 8.0	33.0 ± 2.0	112.0 ± 6.8
‘slow’	‘slow’	6.3 ± 1.7	35.8 ± 9.6	35.6 ± 3.4	120.9 ± 11.7
untreated		17.7 ± 1.6	100.0 ± 8.9	29.5 ± 1.3	100.0 ± 4.5

Material treatment: a/freezing: ‘fast’—liquid nitrogen, ‘slow’—cooling 2°C/min; b/thawing: ‘fast’—immersing in 4°C water, ‘slow’—placing in 4°C air. Percentage values represent amounts of S-HBsAg compared to untreated reference sample.

**Table 1.** S-HBsAg contents in transgenic lettuce leaves after the freeze-thawing cycle [46].

## 6. Optimisation of the lyophilisation process of plant tissue containing S-HBsAg

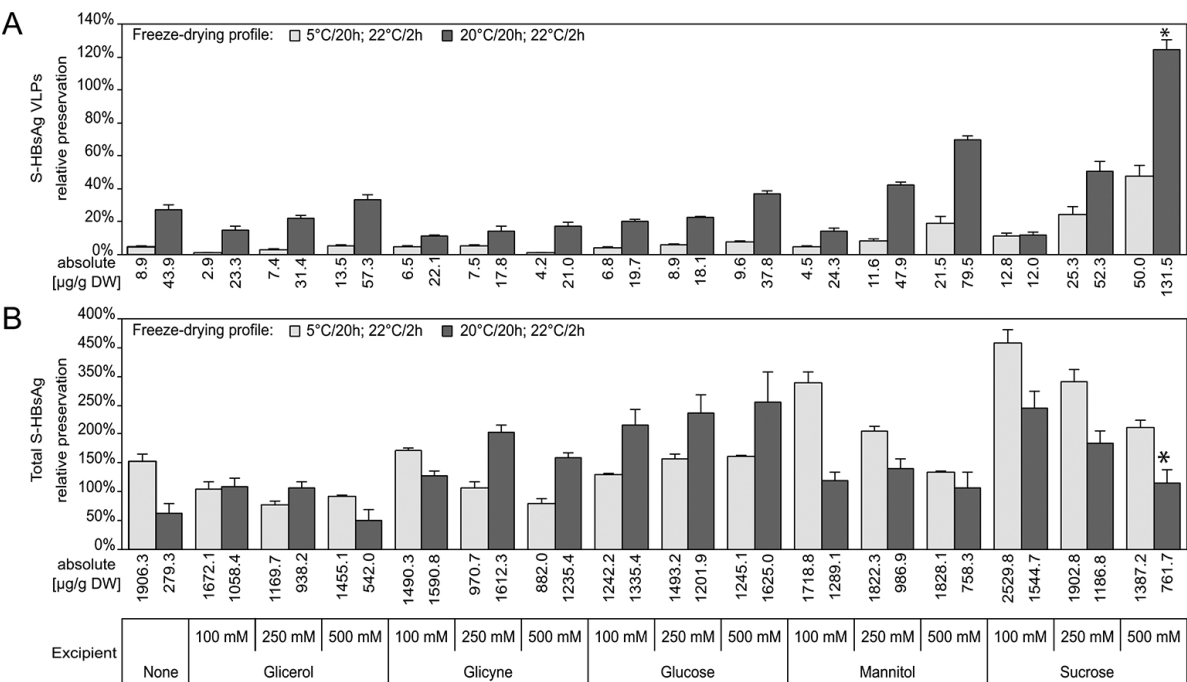
Previously obtained results [10, 11] and the present experiments on freeze-drying and storage of plant-associated S-, M- and L-HBsAg demonstrated the need to optimise lyophilisation. Among tested antigens, the native structure of VLPs was most effectively preserved for S-HBsAg, together with a smaller increase in the total antigen pool, which also includes products of VLPs decomposition. Utilisation of protective excipients, especially sucrose, significantly increased stability of the antigen (**Figures 2–4**). Further optimisation was aimed at determining the most effective freeze-drying conditions comprising a protective additive and physical parameters to ensure the highest stability of S-HBsAg VLPs during the process and the subsequent storage.

### 6.1. Stability of S-HBsAg during lyophilisation in the presence of protective substances

The next stage of research was to study in detail pre-selected substances in terms of stabilising S-HBsAg in order to determine the main/basic excipient—which could be used alone or in

combination with other protective agents. In addition to the previously applied sucrose, mannitol and glycerol, the set of excipients was extended to include glycine and glucose, all used in a wider range of concentrations—100, 250 and 500 mM. This selection was performed on the basis of the reported protection efficacy [13, 16–18, 20, 22, 42, 43] and taking into consideration requirements for the oral route of formulation delivery, that is, harmless ingestion and low cost for product scalability. Glycine was considered as a good crystalline substance with an ability to stabilise the microenvironment of lyophilised proteins [20, 42]. In contrast, glucose, despite the frequently occurring adverse Maillard reaction, was used because of its high ability to penetrate and adhere to protein surface [17]. Initially, two drying profiles were used—20-h primary drying at 5 or 20°C, combined with secondary drying at 22°C for 2 h, identical for the two variants (in short: 5°C/20h–22°C/2h and 20°C/20h–22°C/2h).

The experiments showed that drying at a higher temperature resulted in a much higher degree of VLPs conservation in all excipient variants and for untreated tissue (**Figure 5**). For most of the added substances, a relatively low preservation of VLPs was observed in parallel with large fluctuations in the total antigen pool. However, considerable protective properties were expressed by sucrose and mannitol at concentrations of 250 and 500 mM. Moreover, good stability of VLPs was accompanied by moderate changes in the total antigen level. In particular, the variant with tissue saturated with 500 mM sucrose solution and freeze-dried at 20°C was significantly more efficient than the other options. However, a rise by 24% was observed in the level of VLPs above the baseline level, which most likely resulted from partial dissociation



**Figure 5.** Efficiency of freeze-drying represented as preservation of S-HBsAg VLPs (A) and total antigen pool (B) in lettuce leaves soaked with a series of excipients and processed under two temperature profiles. Preservation effectiveness [%] was calculated as the ratio of VLP-formed and total S-HBsAg in the powdered lyophilised product to the antigen content in fresh tissue multiplied by weight loss degree. The asterisk indicates the variant selected for further experiments [46].

of native particles into smaller 'subparticles' or antigen aggregates. An additional positive effect was the maintenance of the total antigen pool with only small and statistically insignificant changes. This allowed us to draw a conclusion that VLPs disintegration occurred to a relatively small extent and consequently, sucrose was identified as the most promising excipient for further studies. Its use made it possible to achieve significantly higher final absolute antigen contents in the form of VLPs as compared to previous works—approximately 131.5  $\mu\text{g/g}$  DW versus 11–12  $\mu\text{g/g}$  DW [10].

In addition to the determination of S-HBsAg protection effectiveness of individual excipients, a clear trend of an increasing stability of VLPs with increasing concentrations of used substance was observed. However, it was opposed to the behaviour of total antigen, especially in the case of sucrose and mannitol. Because the preferred vaccine formulation would provide a smaller increase in the 'free' antigen pool, the effect was considered favourable.

To summarise this stage of the study, among the analysed protective additives, sucrose proved to be the most effective for the preservation of S-HBsAg VLPs, and at the same time, its use was beneficial by maintaining the total antigen level mostly unchanged. Hence, this compound was selected as the basis for further studies.

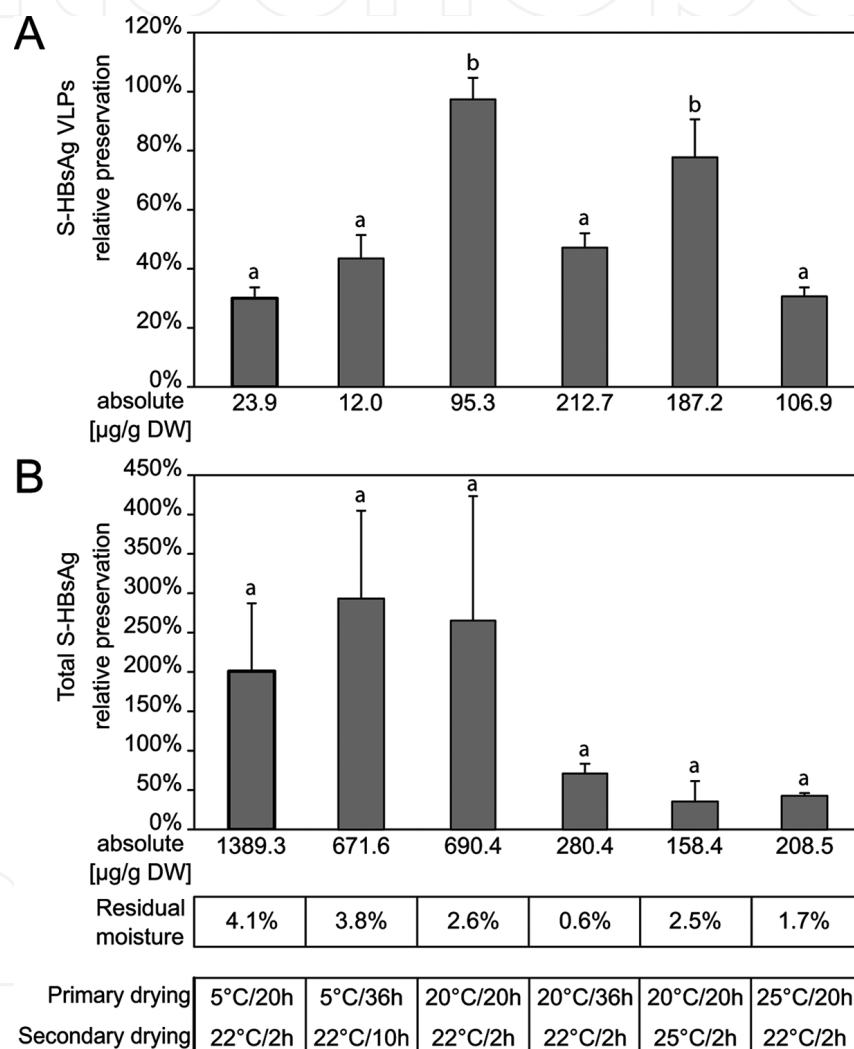
## 6.2. Optimisation of temperature profile

The successive step was focused on determining shelf temperature and duration of freeze-drying, which are of fundamental importance for process economy and scaling up. A higher drying temperature makes it possible to shorten cycle duration and consequently provides a more cost-effective process; however, the material cannot be overheated, as collapse may occur [16, 17]. Also after-process residual moisture is an important factor, having a significant influence on protein recovery.

Results obtained so far indicated that stability of S-HBsAg VLPs was significantly higher when the profile with 20°C for the primary drying temperature was used. Research at this stage focused on attempts to establish a precise drying temperature and time, by the gradual increase in these parameters for primary and secondary drying. Repeated tests of six drying profiles were conducted and they were combined with an analysis of post-process residual moisture (RM) (**Figure 6**).

Freeze-drying with the primary stage conducted at 5°C confirmed a much lower VLPs preservation—approximately 30%, in comparison to processing at 20°C—with an average 97% of retained VLPs in a series of several repetitions. This may have been connected with a high residual moisture in the preparation obtained after drying at a lower temperature—4.1 versus 2.6%. Extension of primary drying to 36 h with an unchanged temperature (5°C) resulted in only slightly decreased RM (3.8%). Although some increase in VLPs stability was visible, it was still insignificant and connected with an enlarged pool of total antigen. In turn, when prolonged primary drying, that is, 36 h at 20°C, was applied and RM was reduced to only 0.6%, it resulted in a significant drop of VLPs and total S-HBsAg. This further confirmed the importance of adequate RM for antigen stability. Next, it was assessed whether shelf temperature beyond 20 and 22°C can be applied. When secondary drying was conducted at 25°C,

2.5% RM was obtained, but VLPs were preserved with a much lower efficiency. In addition, a sharp drop of the total S-HBsAg occurred, probably as a result of the polypeptide chain damage on the VLPs surface. Increasing the temperature of primary drying to 25°C also yielded poor recovery of total and VLP-formed antigen, which could be the result of excessive drying conditions and too low RM. Eventually, the initial profile proved to be the most efficient, indicating that drying temperatures cannot exceed 20 and 22°C, while final RM should be approximately 2.6%. This profile made it possible to save around 100% of VLPs with a relatively moderate increase in the total antigen content.



**Figure 6.** Preservation of VLP-formed (A) and total (B) S-HBsAg in plant tissue lyophilised under different temperature profiles. Sucrose at 500 mM was used as excipient in all variants. Letter indexes mark statistically homogenous groups, separately for the total and VLP-assembled S-HBsAg [46].

In summary, obtained results clearly indicated that for the stabilisation of the appropriate (native) structure of S-HBsAg, both the VLPs and dimers, a certain amount of bound water is required. The water content in the final formulation cannot be too high, since this may favour decomposition processes by enzymatic or physicochemical routes. However, water plays an

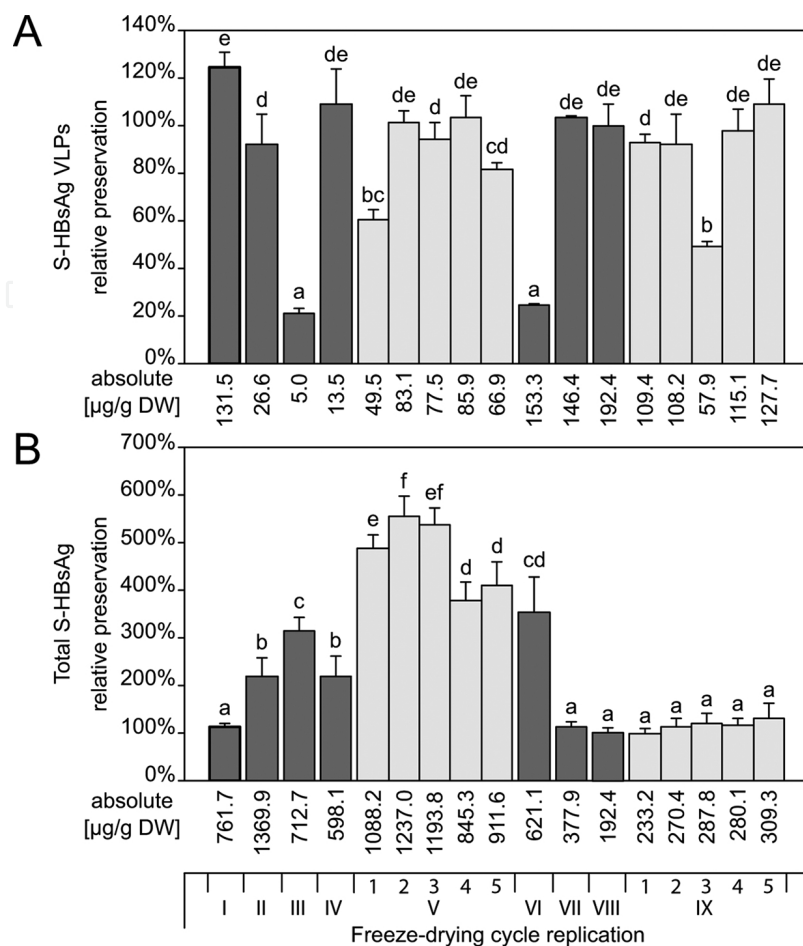


important structural role. Thus, its content cannot fall below a specific critical value, as this can cause VLPs dissociation and ultimately, degradation of the separate antigen molecules due to the complete loss of the hydration shell. Maintenance of an optimum moisture content at 2.5–2.6% was necessary and possible to establish by maintaining an appropriate temperature and time of lyophilisation.

### 6.3. Reproducibility of lyophilisation process and batch-to-batch variation

In previous experiments, it was found that the lyophilisation process with the highest efficiency expressed as the highest degree of VLPs conservation, and simultaneously, the smallest shift in the total antigen pool required 500 mM sucrose as the protective excipient and the drying profile of 20°C/22h–22°C/2h. However, the lyophilisation process is a turn-based method; hence, a batch-to-batch variation does occur [16, 43]. Therefore, in the next part of the study, this aspect was investigated in a series of nine repetitions (I–IX), two of which (V and IX) were divided into separate five independently prepared batches but processed during the same single drying cycle (**Figure 7**). The preservation of VLPs in most cases reached approximately 100%, while only in three batches it was less than 50%. It should be stressed that one of those (IX-3) was one out of five homogenous repetitions tested in a single freeze-drying process. The observed fluctuations confirmed a natural process variation. Nevertheless, overall freeze-drying consistency was considered acceptable, as 13 out of 17 batches resulted in an almost complete VLPs preservation. Overall efficiency for all tested batches was 86%, while it was 97% for batches where VLPs preservation reached minimum 50%.

The process reproducibility in relation to the total antigen pool was much more ambiguous. Observed levels of ‘free’ antigen after lyophilisation ranged from 98% to as much as 556% as related to the theoretical, complete preservation of S-HBsAg and showed no clear connection with VLPs preservation or degradation. The differences in effectiveness were observed between separate processes and during the single drying cycle as in batch V. In some cases, a significant apparent increase in the total amount of antigen was associated with the breakdown of VLPs, as in batches III or VI. In other cases, despite an approximately 100% preservation of VLPs, the level of total antigen increased dramatically, for example in batch V. A possible explanation of this variation during freeze-drying might be that even a partial destruction of VLPs could result in a high increase in the total antigen content, similarly as in freeze-thawing. Even though VLPs are effectively preserved during lyophilisation, some particles may still disintegrate and the released S-HBsAg dimers add to the total antigen pool. However, relatively mild process conditions caused low degradation of S-HBsAg polypeptide chains and resulted in an increased total antigen level. However, in most cases, both VLP-formed and ‘free’ antigen, remained relatively stable—around 100% of preservation, in particular in repetitions I and VII–IX. It is worth noting here that the total antigen level never fell below 100% in any batch, indicating that the optimised freeze-drying process ensured inhibition of S-HBsAg degradation processes at the molecular level. Despite fluctuations of the total antigen level, it can be said that the key is to achieve high reproducibility of the process in relation to VLPs as the specific immunogen.



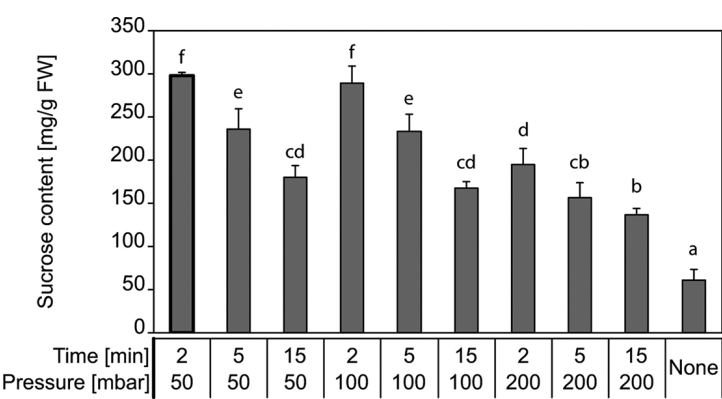
**Figure 7.** Preservation of S-HBsAg—VLP-formed (A) and total (B)—during freeze-drying replication tests. Each batch was run under the profile of 20°C/22h–22°C/2h and with 500 mM sucrose as the excipient. Batches V and IX were loaded in five separate samples. Letter indexes mark statistically homogenous groups, separately for the total and VLP-assembled S-HBsAg [46].

The absolute content of S-HBsAg in obtained preparations varied significantly, as it directly depended on the antigen expression level in processed plant tissue. VLPs contents ranged from 5.0 to 192.4 μg/g DW, while the content of total antigen in lyophilisate ranged from 196.2 to 1369.9 μg/g DW. However, obtained absolute values of the total S-HBsAg pool were considerably lower than previously reported, amounting to approximately 5000 μg/g DW [10]. Summarising, the elaborated freeze-drying process expressed satisfactory reproducibility regarding S-HBsAg preservation. Furthermore, obtained material was characterised by high VLPs contents along with the total antigen level being not excessive, which is considered beneficial in terms of oral vaccination.

#### 6.4. Freeze-drying-associated processing

Although freeze-drying was the crucial stage in plant tissue processing, the associated handling steps such as tissue infiltration with a lyoprotectant and milling of the product could also affect the final efficiency of the whole process. Hence, both steps had to be closely

investigated. Literature data and our results showed that the preservation effect was associated with a high concentration of the protective excipient, here 500 mM sucrose [17, 20] (**Figures 1 and 5**). Therefore, maximal effectiveness of lyoprotectant infiltration had to be ensured. Time (2–15 min) and pressure (50–200 mbar) of soaking were tested (**Figure 8**). The content of S-HBsAg was assayed after soaking and showed no fluctuations in tested parameters. Interestingly, the shortest soaking time proved to be the most effective in all variants, whereas under vacuum of 50 mbar sucrose concentration in tissue was the highest. However, soaking under 100 mbar was only minimally lower and statistically equal. Most likely this lower pressure during soaking created a bigger pressure differential between the chamber and the interior of the leaf tissue, leading to a deeper and more extensive penetration of tissue by the excipient solution. In contrast, prolonged treatment may have resulted in a gradual equalisation of pressure between the chamber and the interior of plant cells, causing greater damage of cells and tissue, and consequently providing a gateway of the excipient. For further work, the variant employing 2-min time and 100 mbar pressure as final parameters was selected due to the milder processing conditions.



**Figure 8.** Sucrose concentration after different soaking conditions in lettuce leaf tissue. Letter indexes mark statistically homogenous groups [46].

Lyophilised tissue had to be milled to obtain the final semiproduct. Performed tests showed that within a period of 5–90 seconds, no significant differences occurred regarding both VLPs and total S-HBsAg contents (data not shown). Yet, since approximately 20 seconds were enough to mill the tissue completely, this time was adopted.

**7. Storage optimisation of freeze-dried plant material containing S-HBsAg**

Initial studies on long-term storage of freeze-dried leaf tissue made it possible to establish that the use of sucrose as an excipient ensures relative stability of VLP-formed S-HBsAg at 4°C, in comparison to other variants [55]. However, VLPs still underwent gradual degradation (approximately 30%). In addition, cold storage is disadvantageous economically and is also in contradiction to the postulated wide availability of a plant-made vaccine. Therefore, research on improving the stability of VLPs in the lyophilised plant material was continued.

A number of physical and chemical pathways of protein degradation may occur during storage in solid states, with almost all being promoted with increasing temperature. The most common and probable pathways comprise a shift of molecular mobility, oxidative and water activity and protein aggregation. Although mobility by principle is limited in the glassy state, on pharmaceutical time scales, it may be sufficient for a considerable molecular mobility of pharmaceutical solids at temperatures up to 50°C below their glass transition temperature [27, 56]. For sucrose, this point is estimated at 60°C in the solid state, and exceeding this threshold during storage at over 10°C could promote collapse of amorphous sucrose [20]. To improve macroscopic mechanical properties of the final product by forming stronger dried cakes and provide support for the amorphous phase, bulking agents, such as mannitol or glycine, may be added. Glycine is widely employed having several advantages, including non-toxicity, high solubility, high eutectic temperature and easy crystallisation [38, 42, 57].

The content of residual water in the product cake throughout its shelf life may be the most important factor responsible for instability [20]. Changes in water reactivity caused by temperature increase, or its content due to transfer from the vial stopper may plasticise the structure of the product cake, thus increasing molecular mobility and promoting various reaction pathways [27, 58]. For this reason, an addition of bulking agents and/or desiccants may have positive effects [44, 59].

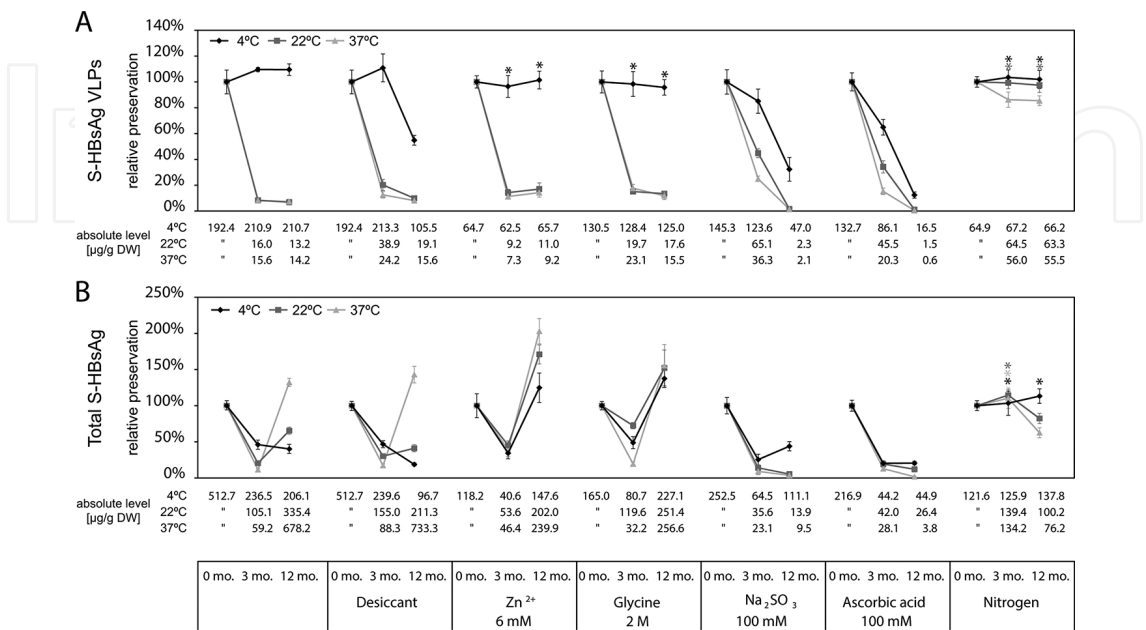
Oxidation may be the next major degradation factor for therapeutic proteins and peptides [27, 41, 60]. Despite the complicated and poorly understood theoretical aspects of oxidation processes in dried protein formulations, this degradation can be minimised by sealing vials under neutral atmosphere and/or using chemicals which compete with protein functional groups and bonds for possible oxidisers, for example, reactive oxygen species (ROS). Such antioxidants as sodium sulphite ( $\text{Na}_2\text{SO}_3$ ) and ascorbic acid are commonly used as preservatives in the food industry.

In addition to well-known protective agents, several others were also recognised to beneficially affect stability of lyophilised proteins, for instance divalent metal ions, especially zinc [61, 62]. However, mechanisms of their action remain unknown.

Apart from the above-mentioned factors, protein aggregation may have the most essential impact on antigen stability, although reasons of this process are still under investigation [63]. Protein aggregation may take place on physical and/or chemical levels, and it clearly depends on the nature of the protein studied. Currently, the dominant view on aggregation is that the main effort needs to be focused on solutions and rather than trying to prevent protein association, we should avoid or counter conformational alteration in the first place [60]. As aggregation poses a significant threat to freeze-dried formulations, in the case of S-HBsAg, this was not encountered. In fact, disaggregation of VLPs appeared to be the crucial issue in our studies. Therefore, the main effort had to be focused on stabilisation of this antigen form.

Long-term storage tests were carried out on preparations obtained applying the process conditions (soaking, freeze-drying and milling) established previously. Apart from the use of only the basic excipient—500 mM sucrose, it was supplemented with additional substances, which included various stabilisers, for example, divalent metal ions— $\text{Zn}^{2+}$ , bulking agents—

glycine, antioxidants—sodium sulphite and/or ascorbic acid, as well as inert atmosphere of nitrogen. All variants tested were used for one year storage at 4, 22 or 37°C in sealed containers, both with and without the presence of silica gel as a desiccant (**Figure 9**).



**Figure 9.** Stability of VLPs (A) and total (B) S-HBsAg in freeze-dried tissue stored for one year at 4, 22 and 37°C. Material was infiltrated with 500 mM sucrose supplemented with different additives and stored under various conditions (with/no desiccant, N<sub>2</sub> atmosphere). Preservation efficiency is represented as a relative change of antigen level to the storage start point. Asterisks indicate insignificant fluctuations in the antigen content with regard to the respective starting point, separately for VLP-assembled and total S-HBsAg.

Most of the tested additional protective substances showed no effect on the efficiency of lyophilisation itself. Preservation of S-HBsAg VLPs and total antigen reached at least 80 and 100%, respectively, which was comparable to the reference material soaked with sucrose alone. Similarly, the moisture content after lyophilisation formulations ranged from 2.6 to 3.0%. Because of the fact that the total amount of the antigen in the lyophilisate directly depended on its content in the initial plant material, storage efficiency was estimated in relation to the starting antigen level immediately after drying, set at 100%. Maintenance of VLPs together with minimal fluctuations of the total antigen level after 3 and 12 months of storage were adopted as determinative in assessing efficacy of the variant.

In almost all tested variants, additional excipients exhibited no enhanced protective effect, since a clear decrease in the VLPs levels was observed, particularly in preparations stored at 22 or 37°C. Total antigen content also fluctuated significantly, indicating that smaller aggregates and dimers released from disassociating VLPs were also subject to severe degradation. In a vast majority of preparations, a degradation scheme was similar to the reference variant, where only sucrose was used.

None of the variants containing zinc ions and/or glycine exhibited a significant improvement in VLPs stabilisation (**Figure 9A**). In turn, the after-process content of the total antigen was



higher than in the reference variant, which indicated a reduced degradation (**Figure 9B**). In these variants, total antigen levels initially dropped, yet later dimers and aggregates released from VLPs were degraded to a lesser degree when compared to other variants, hence the final content increased.

Formulations containing antioxidants, that is  $\text{Na}_2\text{SO}_3$  and/or ascorbic acid, showed initially (for three months) a slower rate of VLPs degradation, both at room temperature and at  $37^\circ\text{C}$ . However, at the end of the storage period (12 months), almost the entire pool of VLPs was degraded (**Figure 9A**). 'Free' S-HBsAg also underwent a strong degradation and the final content of total antigen was significantly lower than in the other variants (**Figure 9B**). This effect could result from the pro-oxidant activity of antioxidants, which occurs when these are excessively concentrated. However, the degradation pattern was very similar not only for 100 mM, but also when 10 mM antioxidant solutions were used. This indicated that even a minimal addition of antioxidants may pose an adverse effect on S-HBsAg.

In turn, the use of a desiccant, in parallel with other additives or treatments, resulted in a similar or more intense process of S-HBsAg degradation for both VLPs and the unstructured antigen. A desiccant added to the formulation containing only sucrose as the protective excipient, or additionally kept under nitrogen atmosphere, caused a significant degradation of both VLPs and the 'free' antigen, particularly during the second storage period—between the 3rd and 12th month (**Figure 9**). An adverse effect of the desiccant effect confirmed an irreplaceable role of residual water in the preservation of integrity of both VLPs and the antigen itself.

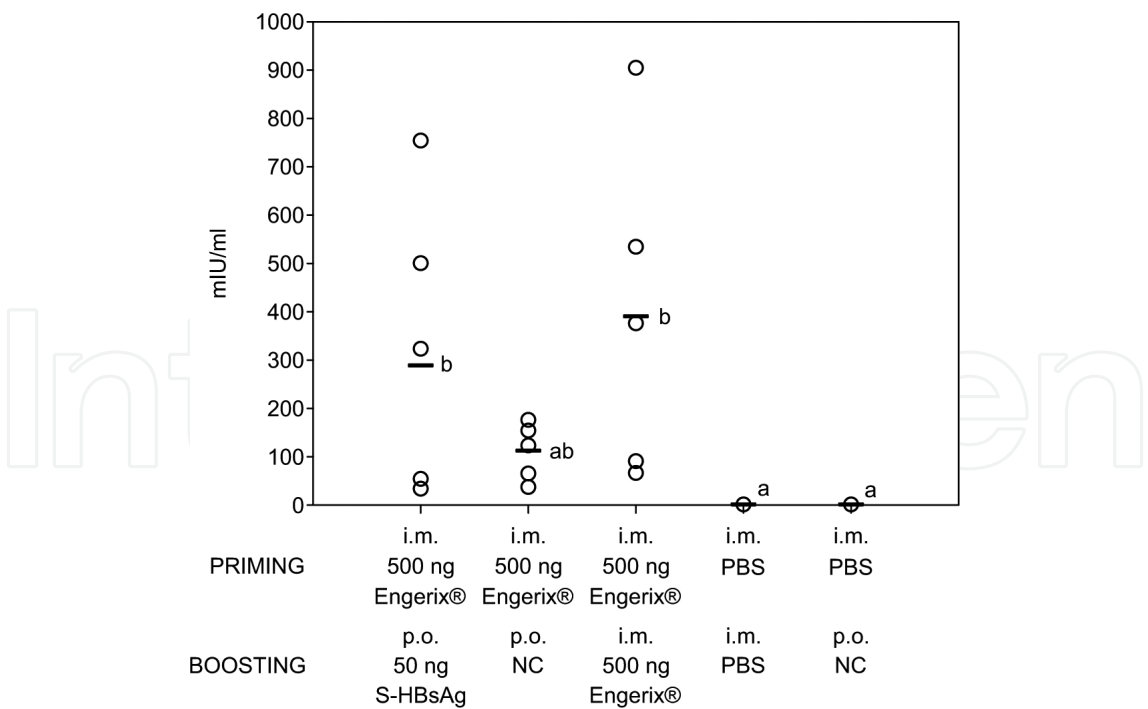
Only in the variant where the lyophilised tissue was soaked solely with sucrose and stored merely under an inert nitrogen atmosphere, S-HBsAg remained stable during long-term storage. The level of VLPs (**Figure 9A**) was unchanged both at cold and at room temperature. Only at  $37^\circ\text{C}$  a small (approximately 15%), statistically significant reduction was observed in the VLPs content. Nonetheless, this occurred only within the first three months of storage, and after that, the level did not change. The content of total antigen (**Figure 9B**) remained completely (statistically) unchanged only when stored in the cold. In contrast, at 22 and  $37^\circ\text{C}$ , its initial stable level eventually decreased substantially, to approximately 30 and 50%, respectively. Considering a potential vaccine formulation, this should not pose major problem, as the VLP-assembled S-HBsAg is the immunisation agent. Moreover, when compared to the other variants, and in particular to the reference sample, nitrogen significantly alleviated the degradation process, which suggests a conclusion that oxidative/reductive activities play the most important role in long-term stability of S-HBsAg, as it is for many other freeze-dried proteins [27, 41, 60].

## 8. Oral immunisation using lyophilised plant tissue-containing S-HBsAg

For the purpose of a prototype plant-derived anti-HBV oral vaccine in the form of freeze-dried plant tissue, apart from maintaining high contents and the native structure of S-HBsAg assembled into VLPs, it was equally important to confirm their immunogenicity. Hence, animal immunisation trials were performed, where mice were vaccinated by intramuscular

(i.m.) priming with the commercial Engerix®B vaccine and subsequently received an oral booster (*per os*, *p.o.*) with a suspension of powdered lyophilised tissue based on the previously developed low-dose protocol [10]. The lyophilisate came from batch No. IV with a 109% preservation rate of S-HBsAg VLPs , corresponding to 29 µg/g DW and 199% or 538 µg/g DW of total antigen. Hence, the dose of 50 ng VLPs was delivered in 1.72 mg of freeze-dried tissue per individual subject.

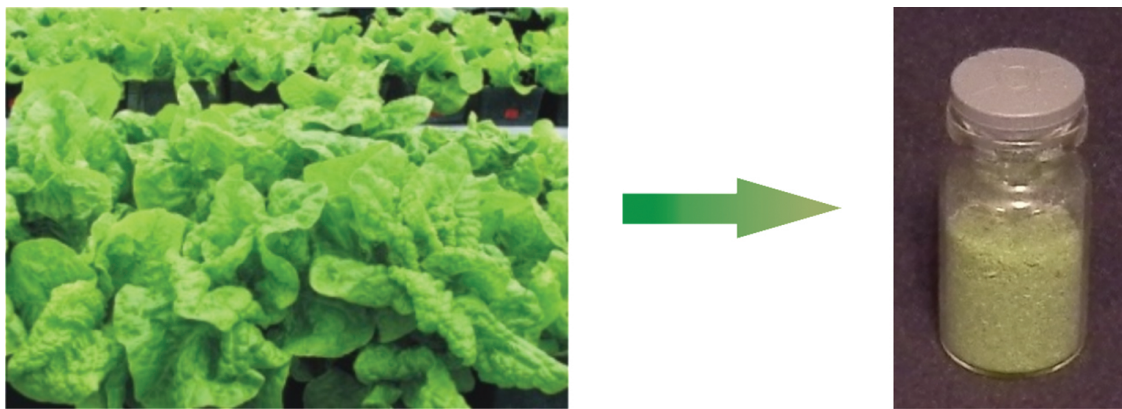
Results of mouse vaccination are summarised in **Figure 10**. The titre of anti-HBs antibodies in mice boosted orally with the lyophilised powder reached a mean value of 293 mIU/ml. Although it was lower, the response was statistically equivalent as in the reference group boosted by an injection with Engerix®B, while the response patterns were also similar. When mice were orally administered the control tissue, no boosting effect was observed. Even though the used freeze-dried preparation exhibited some build-up of total S-HBsAg, apparently it did not hinder immune response development. This result might be obtained due to the considerably lower absolute level of total antigen in the lyophilised tissue than that used previously [10]; however, an exclusively oral immunisation was then performed. Moreover, although the observed response was lower, it was comparable to other reports on injection-oral vaccination trials where plant-associated S-HBsAg was CTB- or LTB- adjuvanted [5, 64]. In conclusion, induction of systemic immune response presented in our study confirmed that the selected parameters of plant material processing ensure successful preservation of S-HBsAg antigenicity and immunogenicity, fundamental to a potential oral vaccine against HBV.



**Figure 10.** Anti-HBs antibody response in mouse sera after oral boosting with S-HBsAg in powdered lyophilised tissue. Material for immunisation was freeze-dried under the 20°C/22h–22°C/2h profile with 500 mM sucrose as a protective excipient. Symbols: ○ individual mouse response; – group mean value; NC - control lyophilisate (negative control). Letter indexes mark statistically homogenous groups [46].

## 9. Conclusion

Presented research confirmed that freeze-drying may be exploited for effective processing of such complex preparations as plant tissues containing antigenic proteins assembled into virus-like particles. Here, we showed consecutive steps of our study on HBs antigens to finally establish a technology for S-HBsAg—the basic antigen of hepatitis B virus. The freeze-drying technology, together with storage under inert nitrogen atmosphere, made it possible to obtain a durable intermediate, having a stable content of immunogenic S-HBsAg VLPs, which could be used in the final formulation—a tablet, capsule, etc.—of an oral vaccine against HBV (**Figure 11**). Although adaptation of this method to a specific protein requires numerous steps and repetitions, it appears fundamentally important for the conversion of plant tissue to a form of an oral vaccine against HBV and most probably also other pathogens.



**Figure 11.** Conversion of lettuce leaf tissue into lyophilised and powdered intermediate with stable S-HBsAg content for preparations of an oral vaccine against HBV.

## Author details

Marcin Czyż and Tomasz Pniewski\*

\*Address all correspondence to: [tpni@igr.poznan.pl](mailto:tpni@igr.poznan.pl)

Institute of Plant Genetics Polish Academy of Sciences, Poznań, Poland

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