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Maltose-mediated, long-term stabilization of freeze- and spray-dried forms of bovine and porcine hemoglobin

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Abstract: Slaughterhouse blood represents a valuable source of hemoglobin, which can be used in the production of heme-iron based supplements for the prevention/treatment of iron-deficiency anemia. In order to obtain a stable solid-state formulation, the effect of maltose addition (30 %) on the stability and storage of bovine and porcine hemoglobin in powders obtained by spray- and freeze-drying (without maltose: Hb; with maltose: HbM) were investigated. Differential scanning calorimetry of spray- and freeze-dried powders indicated satisfying quality of the formulation prepared with maltose on dissolving back into solution. After two-year storage at room temperature (20±5 °C) in solid forms, protected from moisture and light, rehydrated spray- and freeze-dried HbM were red, while Hb were brown. Dynamic light scattering showed the presence of native hemoglobin monomers in rehydrated spray- and freeze-dried HbM, but their agglomerates in Hb samples. UV–Vis spectrophotometry confirmed an absence of significant hemoglobin denaturation and methemoglobin formation in HbM freeze-dried powders. In spray-dried HbM, an increased level of methemoglobin was detected. The results confirmed the stabilizing effect of maltose, and suggested its use in the production of long-term stable solid-state formulations of hemoglobin, along with drying processes optimization.

Keywords: slaughterhouse blood; heme-iron protein; dynamic light scattering; UV–Vis spectroscopy.

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INTRODUCTION

Iron deficiency continues to be the leading cause of anemia worldwide, and has a substantial effect on the quality of people's life in both low-income and developed countries.[1] Most of the commercial products (supplements and drugs) for the prevention and treatment of iron-deficiency anemia are based on non-heme-iron.[2] The necessity for the development of a new, heme-iron supplement is based on the following facts: 1) bioavailability of heme-iron is higher than non-heme iron (20–30 % compared to 3–8 %, respectively); 2) absorption of heme-iron is not affected by other dietary components and 3) heme-iron treatment increases the serum iron level 23 times more than non-heme-iron.[2,3] The main sources of heme-iron are meat and seafood, *i.e.*, hemoglobin and myoglobin as their constituents. In any slaughter, 4 to 5 L of blood per 100 kg of bovine/porcine mass could be obtained.[4,5] Knowing that 1 L of bovine blood contains 110 g of hemoglobin[6] and 0.4 g of heme iron, it was calculated that in a technological process with a yield of 70 % from blood of only one cattle, with an average weight of 500 kg, it is possible to isolate 1.7 kg of hemoglobin protein,[7] containing 6 g of heme iron. Unfortunately, slaughterhouse blood is mainly discarded and treated as a waste exposing a high pollutant capacity.[8] If this blood were properly collected and processed, it could be used to generate high-added-value food ingredients due to its exceptional nutritive value and functional properties.[9] It has already been shown that heme iron-rich blood products improve the iron status of animals[10] and human subjects with anemia.[11–13] From an industrial point of view, it is simpler and more cost effective to transport, store, and handle solids than liquid functional food products.[14] However, the development of a process for hemoglobin isolation and its further conversion to a long-term stable solid state is still a challenge due to the susceptibility of hemoglobin to denaturation.[15] Regarding the techniques for the production of solid forms of active ingredients on the industrial scale, the most commonly used are freeze-drying (lyophilization) and spray-drying.[14] On the other hand, these processes can cause irreversible damage to proteins, manifested as structural denaturation and loss of biological efficacy.[16] A wide variety of agents, including sugars, polyols, amino acids, and other polymers can offer thermodynamic stabilization to proteins in liquids.[16,17] The usage of sugars – the solid state interactions with proteins of which and applications in the food and pharmaceutical industry have been extensively described – might be the most acceptable approach to the design of stable forms[18] of hemoglobin.[19] Nowadays, trehalose is accepted as an exceptional and the most commonly used protein stabilizer.[20] However, the stabilizing effect of lower-priced maltose on proteins has not yet been intensively studied. Chung *et al.*[21] showed that methemoglobin formation in air dried films of human maltose-embedded hemoglobin has been successfully suppressed due to reducible property of maltose. Besides, the retained ability of deoxyhemoglobin film to

convert into oxyhemoglobin suggested the strong and unexploited potential of maltose monohydrate to preserve hemoglobin structure and function.[21] Additionally, maltose possesses the characteristic of relatively fast dissolution, sweet taste and low viscosity, which provide a "smooth melt feeling".[22] In this work, the effect of maltose addition to bovine and porcine hemoglobin solution isolated from slaughterhouse blood (bHb and pHb, respectively) on long-term stability of hemoglobin powders obtained by freeze- and spray-drying was investigated. The produced formulations were characterized using differential scanning calorimetry (DSC), dynamic light scattering (DLS) and UV–Vis spectroscopy.

EXPERIMENTAL

Blood samples, hemoglobin isolation and purification

Porcine and bovine blood was obtained from the slaughterhouse "PKB Imes" in Belgrade, Serbia. Blood collection, transportation and handling were realized according to the protocol given in Kostić *et al*.[23] Hemoglobin (Hb) was isolated by a gradual hypotonic hemolysis process, also described in Kostić *et al.*,[23] but conducted under aseptic conditions. The hemolysates were partially purified by tangential ultrafiltration through filters with pore size 0.2 μm and 100 kDa (Viva Flow® 50, Sartorius AG, Germany). The Hb concentration was determined by cyanmethemoglobin method and then adjusted to 10 g L^{-1}. In order to obtain formulations with maltose, maltose monohydrate was dissolved in the purified hemolysate (below indicated as Hb solution) to a final concentration of 30 %.

Electrophoretic analysis

The protein contents in the Hb solutions before addition of maltose were analyzed by 1) isoelectric focusing (LKB 2117 Multiphor II, LKB Instruments Ltd., UK) on a 7.5 % polyacrylamide gel with 3 % ampholyte solution on a pH gradient from 3.5 to 10 and 2) reducing SDS-PAGE (SE 260 Mighty Small II vertical slab electrophoresis unit (GE HealthCare Life-Science, USA) on 12 % gel. The proteins are visualized by Coomassie brilliant blue staining.

Spray-drying of hemoglobin

Aliquots of bHb and pHb (with or without maltose) were spray-dried using a Büchi Mini spray dryer B-290 (Büchi, Switzerland) according to the protocol of Salvador and co-workers,[24] under the following conditions: inlet temperature 140 °C, outlet temperature 68 °C, and flow rate, 8 mL min^{-1}. The obtained powders were transferred to polyethylene micro tubes which were sealed with parafilm and then kept for two years in the dark, at room temperature (20±5 °C), in silica gel desiccators.

Freeze-drying of hemoglobin

Aliquots of bHb and pHb (with or without maltose) were lyophilized in Petri dishes with BETA 1-8 LD plus lyophylizator (Martin Christ, Germany). After cooling to –70 °C, the Petri dishes were transferred to a shelf of a freeze-drying apparatus. The primary drying was conducted with a shelf temperature of –60 °C for 24 h followed by –65 °C for 2 h. The obtained samples were kept the same way as described for the spray-dried samples.

Differential scanning calorimetry (DSC)

DSC was used to measure the basic thermostability or "susceptibility" of the proteins to thermal denaturation.[25] DSC aluminum pans (30 μl, D. 6.7 mm×3 mm, 08/HBB37408) with 10–12 mg of the samples were hermetically sealed and analyzed using a DSC131 Evo (Set-

aram Instrument, Caluire, France), previously calibrated with indium. An empty sealed pan was used as a reference. Both pans were placed in a chamber, kept at 30 °C for 5 min and subsequently heated from 30 to 110 °C at a constant heating rate of 2 °C min^{-1}. The nitrogen-flow was 20 mL min^{-1}.

UV–Vis spectroscopy

The UV–Vis absorption spectra of the Hb samples were recorded using a UV-1800 UV––Vis spectrophotometer (Shimadzu, Japan). Before analysis, solid forms of Hb were reconstituted in PBS and centrifuged for 10 min at 800*g*, at 4 °C and the absorbance at 415 nm was adjusted to 1 by diluting the samples with PBS.

Dynamic light scattering (DLS)

The size distribution based on particles number of the bHb and pHb preparations was analyzed by DLS using a Malvern zetasizer Nano ZS (Malvern Instruments, UK). Prior to analysis, solid forms of Hb were dissolved in 0.22 μm filtered PBS and centrifuged 10 min at 800*g*, at 4 °C. All measurements were repeated three times.

Morphological examination of Hb solid forms

Morphological examination of the obtained hemoglobin solid forms by field-emission scanning electron microscopy (FE-SEM) was performed as described in Kostić *et al.*[23]

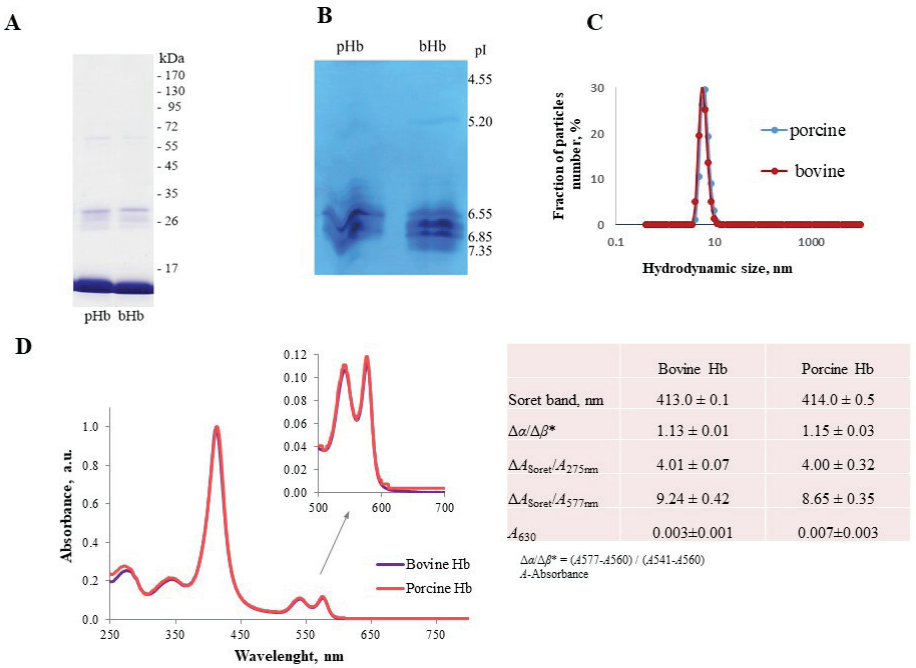
Statistics

Statistical analysis was performed with the use of Microsoft Office Excel 2007 software (Microsoft Corporation, WA, USA). Differences between groups were tested for statistical significance ($p < 0.05$) by the Student's *t*-test.

RESULTS AND DISCUSSION

Prior to the production of the solid Hb formulations, the purity of starting porcine and bovine Hb solutions was estimated by SDS-PAGE and isoelectric focusing (Fig. 1A and B). As can be seen from Fig. 1A, SDS-PAGE run under reducing condition revealed that 91±2 % of the total proteins represent globin chains at 16 kDa. Besides this 16 kDa band, two weak protein bands with apparent molecular masses of 30 and 70 kDa were found in the prepared Hb solutions. Isoelectric focusing showed the existence of two intensive and several weak Hb bands at isoelectric points (pI) from 6.5 to 7.3.[26,27] A weak protein band at pI 5.85 was also present (Fig. 1B). These data revealed relatively high level of protein purity, even though no additional purification technique, such as chromatography, was used. DLS analysis (Fig. 1C) demonstrated the presence of Hb molecules with hydrodynamic diameter of ≈7 nm, which corresponds to hydrodynamic diameter of native hemoglobin monomer (*i.e.*, α2β2 globin tetramer),[28] without presence of aggregates or decomposed molecules. UV–Vis absorption spectroscopy has been employed as a universal method for investigating structural changes of proteins.[29] Absorption bands at 275, 350, 413–415, 541, 576 and 630 nm, characteristic for native oxyhemoglobin, were identified (Fig. 1D). The absorption maximum at 275 nm originates from the aromatic heterocyclic rings of tryptophan and tyrosine residues[30] from the globin chains. The coordinate covalent bond between iron and the globin chains *via* proximal histidine is res-

ponsible for the absorption maximum at 350 nm, while porphyrin ring of the heme group shows an intensive absorption maximum at 413–415 nm, *i.e.*, the Soret band.[31] The two absorption maxima at 541 and 577 nm (α and β) derived from oxyhemoglobin[32] were also detected. The absorbance at 630 nm, originating from methemoglobin,[32] was very low. Besides, the UV–Vis spectra analysis showed that minimal individual variation in Hb molecules existed (inset Table in Fig. 1D), which allowed solid formulation from pooled samples to be prepared.



	Bovine Hb	Porcine Hb
Soret band, nm	413.0 ± 0.1	414.0 ± 0.5
$\Delta\alpha/\Delta\beta$*	1.13 ± 0.01	1.15 ± 0.03
$\Delta A_{Soret}/A_{275nm}$	4.01 ± 0.07	4.00 ± 0.32
$\Delta A_{Soret}/A_{577nm}$	9.24 ± 0.42	8.65 ± 0.35
A_{630}	0.003±0.001	0.007±0.003

$\Delta\alpha/\Delta\beta^* = (A577-A560) / (A541-A560)$
A-Absorbance

Fig. 1. Physicochemical characterization of bovine (bHb) and porcine hemoglobin (pHb) solutions isolated from slaughterhouse blood by gradual hypotonic hemolysis and purified by tangential ultrafiltration: A, SDS-PAGE under reducing conditions (sample: 25 μg protein); B, isoelectric focusing (sample: 100 μg protein); C, size distribution by particles number; D, representative UV–Vis spectra. Data in the inset table represent mean ±*SD* of four different hemoglobin samples of both species.

Spray- and freeze-drying are the two most frequently used methods for drying protein solutions in the food and pharmaceutical industries.[33] However, these techniques also have some shortcomings: they cause many destabilizing stresses that could result in irreversible protein denaturation/oxidation,[33] if protective agents are not added.[34] The protective effect of disaccharides such as sucrose and glucose during the spray- and freeze-drying of human and porcine Hb[34] have been described; however data on the efficacy of maltose to mediate the stability of dried hemoglobin by these techniques are scarce. Suppression of methemo-

globin formation in Hb containing maltose has been reported only for human hemoglobin films dried at room temperature (22 °C).[21]

Guided by the optimized protocols for the dehydration of susceptible proteins by spray- and freeze-drying,[24,35] formulations of bovine and porcine hemoglobin with and without maltose were prepared. Sugars are commonly used as stabilizing agents in concentrations of 10–20 mass %.[21,34,36] Nevertheless, a recent study showed that even higher sugar concentrations (up to 43.6 %), still have a measurable effect on protein structure and stability and, more importantly, can shift the mechanism of protein stabilization from preferential exclusion (preferential hydration) to neutral solvation (partial penetration of sugar into the hydration shell region).[19] Accordingly, in this study, ≈1 M maltose (30 %) as bovine and porcine Hb stabilizer during freeze- and spray- drying was examined.

Both spray-dried bHb and pHb appeared as homogeneous fine powders, while freeze-dried samples of Hb were slightly clumpy powders. Maltose addition during spray-drying resulted in reddish blush like color. In the freeze-dried form of Hb, the red color remained more prominent in the presence of maltose when compared to spray-dried powders (Fig. 2A and B). Although residual humidity of obtained powders was not determined, the spray-dried powders were obviously much less hygroscopic than those obtained by freeze- drying, allowing even direct visualization by field emission scanning electron microscopy (FE--SEM, Fig. 2A, inset).

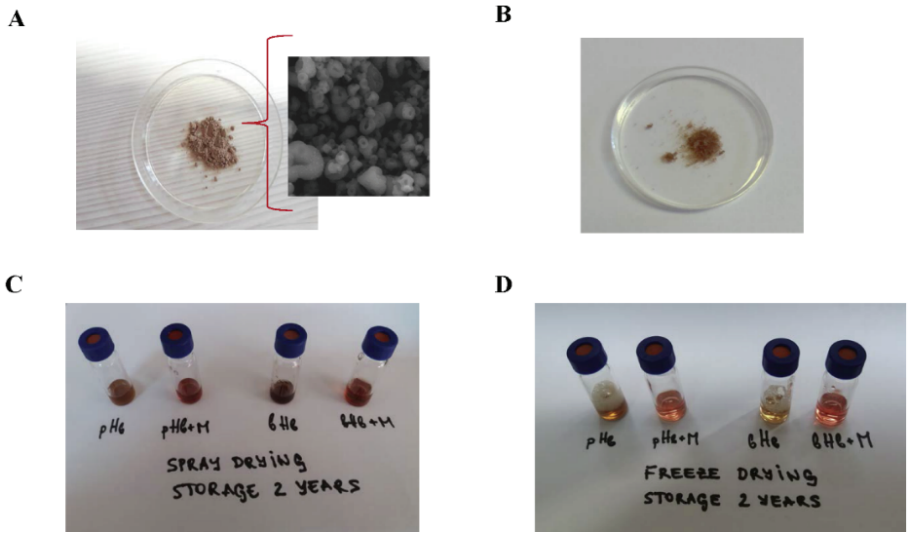


Fig. 2. Representative photographs of: A) spray-dried and freeze-dried bovine Hb with maltose (inset represent FE-SEM micrograph of spray-dried bovine Hb with maltose); B) reconstituted spray-dried and freeze-dried bovine and porcine Hb without or with maltose (bHb, pHb, bHbM and pHbM) after two years of storage at room temperature.

Thermal unfolding and the denaturation profile of maltose monohydrate and pHb and bHb solid formulations are presented in the Supplementary Material to this paper as Figs. S-1–S-3, respectively. The sample of maltose (Fig. S-1) showed a typical endothermic glass transition according to literature data with glass transition temperature T_g = 122 °C,[37] C_p = 11.5 J g^{-1} K^{-1} at T_g and ΔH_{relax} = = 100.36 J g^{-1}. Thermogram of bHb and pHb formulations obtained by freeze--drying (Fig. S-2A and C) demonstrated only one nearly symmetrical endothermic peak with no detectable shoulders or minor peaks, indicating that these samples are thermally homogenous.[38] The T_m value as an indicator of thermal stability and the T_o value as a starting point of denaturation, indicated significant shifting toward higher values for bHb and pHb freeze-dried formulations with maltose (Fig. S-2B and D). The DSC thermograms of spray-dried bHbM and pHbM formulations revealed the existence of peaks with two "shoulders" (Fig. S-3B and D). This is commonly seen in DSC analysis of mixtures, but can also indicate some deteriorating effects of the spray-drying process on Hb stability. However, since the T_m value is an established parameter that can indicate potential shelf life of proteins in pharmaceutical formulation,[39] the DSC analysis preliminarily indicated satisfactory viability of the Hb formulations with maltose on their redissolution.[35]

Dried Hb samples, both with and without maltose, dissolved easily after two years storage. However, the reconstituted spray- and freeze-dried forms of bHbM and pHbM showed significant differences even by visual examination in comparison to the samples without maltose. Formulations with maltose possessed a bright red color, the same as the starting Hb solutions, while reconstituted powders produced without maltose were brown (Fig. 2C and D).

The DLS results (Fig. 3A and C) showed that the hydrodynamic diameters of the reconstituted stored pHbM were 6.19±1.41 nm and 6.97±1.49 nm after freeze- and spray-drying, respectively. In the case of bHb, the hydrodynamic diameter of the samples prepared with maltose also remained unchanged over storage of two years, having diameters of 6.06±1.32 nm and 6.10±1.40 nm after freeze- and spray-drying, respectively. These data indicated that Hb in the presence of maltose remained in the form of native protein (*i.e.*, undecomposed and non-aggregated α2β2 globin tetramer),[40] regardless of the used drying method, and that good stability of the Hb complex with maltose was stable over two-year storage. On the other hand, rehydrated bHb and pHb prepared by these two drying techniques without the addition of maltose revealed the presence of agglomerates with hydrodynamic diameter of 200–500 nm (Fig. 3B and D).

The absorption spectra of different dried forms of pHb and bHb rehydrated after being stored for 2 years at room temperature (20±5 °C) and protected from moisture and light are shown in Fig. 4. In order to assess the ability of maltose to stabilize Hb molecules dried by these two methods, data on absorption spectra of

these rehydrated formulations were compared to our laboratory "standard", *i.e.*, Hb solutions kept at –20 °C for the same period.

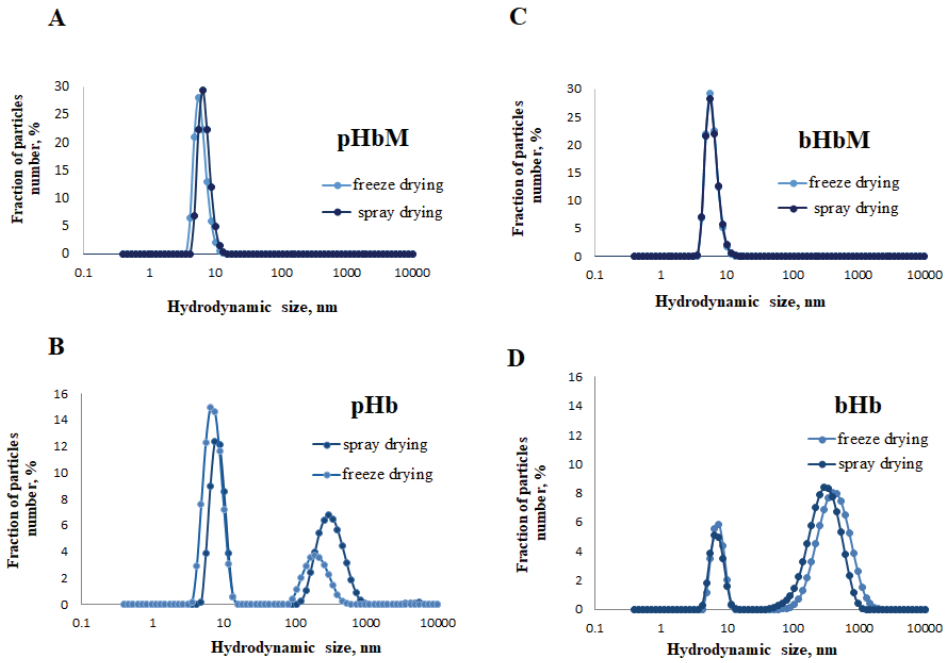


Fig. 3. Number based DLS of reconstituted bovine and porcine hemoglobin spray- and freeze--dried with maltose (bHbM and pHbM) and without maltose (bHb and pHb) after two years storage at room temperature.

The heme group is hidden in the hydrophobic cavity formed by protein chains to avoid the entry of polar molecules or oxidizing agents to protect its stability.[29] Thus, the absorption maximum that originates from heme is an indicator of the deterioration of Hb.[41]

As could be seen from Fig. 4, only slight hypsochromic shifting of the Soret band was detected when the stored Hb formulations with maltose were compared with a fresh sample kept at –20 °C. The values of $\Delta\alpha/\Delta\beta$ ratio close to 1 of both spray- and freeze-dried Hb with maltose (Table S-I of the Supplementary material) confirmed the low level of transformation of oxygenated to oxidized Hb during storage.[31] This result indicated relatively high quality of maltose dried Hb forms stored for two years at ambient temperature protected from moisture and light.[41] A large increase in the A_{Soret}/A_{275} ratio and a large decrease in the A_{Soret}/A_{577} ratio, which reflect a considerable presence of free heme and the breakdown of Hb molecules, respectively,[31] were not detected for freeze-dried Hb with maltose (Table S-I).

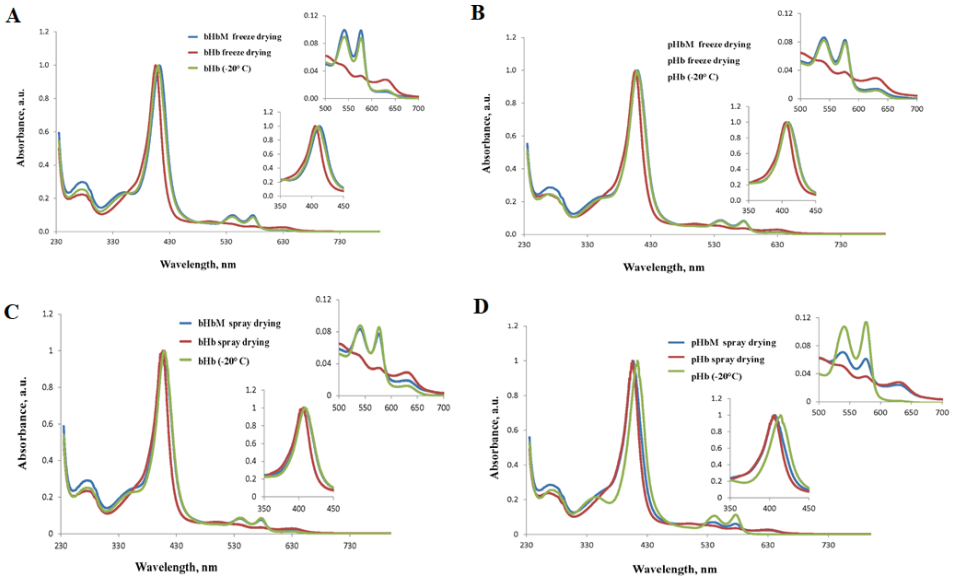


Fig. 4. Representative UV–Vis spectra of freeze-dried (A and B) and spray-dried (C and D) bovine and porcine hemoglobin without (bHb and pHb) and with maltose (bHbM and pHbM) rehydrated after being stored for 2 years at ambient temperature.

Very high A_{Soret}/A_{577} values for formulations with maltose probably reflect intensive Hb degradation through unstable Hb intermediates (ferryl/ferryl radical) that oxidize residues within globin chains and lead to Hb degradation.[42]

Comparable quality of maltose dried Hb forms with Hb solution stored at –20 °C (Table S-I) opens the possibility to avoid storage in freezers, which is impractical (or more precisely, almost impossible) on a large scale. During spray--drying, as well as freeze-drying, substantial oxidation of Hb to methemoglobin occurred in the absence of maltose (Fig. 4C and D and Table S-I of the Supplementary material).

At a given concentration, maltose suppressed methemoglobin formation in bovine and porcine samples more effectively during freeze-drying than in spray--drying (Fig. 4A and B and Table S-I). Similar results were demonstrated by Labrude *et al.*[34] in the case of sucrose usage as a protectant for spray-dried human Hb. This was somewhat expected since spray-drying as dehydration process includes exposition of sample to higher temperature than in the freeze-drying process, accelerating Hb autoxidation and easier access of oxidizing agents to the heme pocket.[24] Several hypotheses have been suggested to explain the protective effect of sugars on proteins.[19,36] According to the "water replacement hypothesis", sugars hydrogen-bond to biomolecules during dehydration, acting as substitutes of hydration water molecules,[19,43] especially when high concentrat-

ions of sugars (>20 %) are used.[19] The "preferential hydration" hypothesis suggests that sugars, rather than directly binding to biomolecules, entrap the residual water at the interface by glass formation, thus preserving the native solvation.[16] Whereas, the third "high viscosity" hypothesis considers the large viscosity of the host glassy matrix responsible for the protection of low-water systems since it causes motional inhibition, hindering of the dynamic process.[44] Probably all these mechanisms contribute to preservation of the native structure of pHb and bHb after their conversion to solid forms by freeze- and spray-drying in the presence of maltose.

CONCLUSIONS

In the present study, the impact of the sugar maltose on the stability of bovine and porcine hemoglobin in the solid state obtained by spray- and freeze-drying was assessed. DSC showed that maltose addition to the hemoglobin solution shifted the starting point of thermally induced denaturation and melting point of dried hemoglobin forms toward higher values, confirming its protective effect. After storage for two years at room temperature (20±5 °C), protected from moisture and light, reconstituted solid formulations of bovine and porcine hemoglobin with maltose retained their color and physicochemical characteristics to those of the respective starting hemoglobin solutions and demonstrated the absence of biologically inactive methemoglobin, as verified by DLS and UV–Vis spectroscopy. Although encouraging, these preliminary results on maltose used as a stabilizing additive indicate the need for more specific optimization of parameters of the drying processes themselves, with the aim of producing long-term stable solid-state formulations of hemoglobin.

SUPPLEMENTARY MATERIAL

Additional data available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ДУГОТРАЈНА СТАБИЛИЗАЦИЈА СУШЕНОГ РАСПРШИВАЊЕМ И ЛИОФИЛИЗОВАНОГ ГОВЕЂЕГ И СВИЊСКОГ ХЕМОГЛОБИНА МАЛТОЗОМ

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Хемоглобин из еритроцита добијених из отпадне кланичне крви је добро познат, али недовољно искоришћен, богат извор хемског гвожђа. У циљу добијања стабилне формулације овог протеина у чврстом стању, испитан је ефекат додавања малтозе у говеђи и

свињски хемоглобин изолован из кланичне крви током сушења распршивањем и лиофилизације. Ефекат малтозе је процењен на основу резултата анализе добијених прахова након две године складиштења на собној температури (20±5 °C), заштићено од влаге и светлости. Пре складиштења урађена је анализа формулација добијених сушењем распршивањем и лиофилизацијом, а диференцијална скенирајућа калориметрија прелиминарно је указала да се може очекивати задовољавајућа стабилност протеина након реконституције формулација припремљених са малтозом. Након двогодишњег складиштења, реконституисане чврсте форме говеђег и свињског хемоглобина добијене сушењем распрскавањем и лиофилизацијом са малтозом задржале су црвену боју, какву је имао и полазни раствор хемоглобина. Фотонска корелациона спектроскопија рехидрираних формулација са малтозом добијених обема техникама сушења, показала је присуство молекула хемоглобина као мономера без присуства агломерата. UV–Vis спектрофотометрија потврдила је одсуство значајне денатурације хемоглобина и формирања метхемоглобина у формулацијама са малтозом добијених лиофилизацијом, док је у чврстим формама хемоглобина са малтозом осушених распршивањем показала повећано присуство метхемоглобина. Резултати ове студије су потврдили ефекат малтозе као стабилизирајућег адитива и њену потенцијалну употребу у производњи дугорочно стабилних чврстих форми хемоглобина, али указали и на потребу за додатном оптимизацијом параметара процеса сушења.

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