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Addition of Pullulan to Trehalose Glasses Improves the Stability of β-Galactosidase at High Moisture Conditions

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ABSTRACT

Incorporation of therapeutic proteins in a matrix of sugar glass is known to enhance protein stability, yet protection is often lost when exposed to high relative humidity (RH). We hypothesized that especially in these conditions the use of binary glasses of a polysaccharide and disaccharide might yield advantages for protein stability. Therefore, different amounts of the polysaccharide pullulan were introduced in freeze-dried trehalose glasses. In these homogeneous blends, the presence of pullulan above 50 weight % prevented crystallization of trehalose when exposed to high RH. Storage stability testing up to 4 weeks of the model protein β-galactosidase incorporated in pullulan/trehalose blends showed superior behavior of pure trehalose at 30 °C/0% RH, while pullulan/trehalose blends yielded the best stability at 30 °C/56% RH. In conclusion, binary glasses of pullulan and trehalose may provide excellent stability of proteins under storage conditions that may occur in practice, namely high temperature and high RH.

1. Introduction

Proteins are becoming an increasingly relevant class of drugs in today's pharmacotherapy. Due to their size as well as their complex and labile structure, ensuring stability of therapeutic proteins requires a completely different formulation approach than for conventional small molecule drugs (Manning, Chou, Murphy, Payne, & Katayama, 2010; Mitragotri, Burke, & Langer, 2014). Generally, pharmaceutical proteins are formulated as aqueous solutions, which have limited shelf life and often require refrigerated storage and transportation. Next to the costs and inconvenience involved with this so-called cold chain (Saxenian, 2017), proteins are often still subject to degradation. In case of impaired stability of proteins, not only their efficacy may be reduced, but degradation products may also elicit undesired immune reactions, with serious consequences for the patient (Jiskoot et al., 2012; Ratanji et al., 2014). Conversely, by storing proteins in the dry state, the degradation rate may be drastically reduced due to limited molecular mobility (Lai & Topp, 1999). However, the stresses during drying and subsequent storage could still impair their integrity (Mensink, Frijlink, van der Voort Maarschalk, & Hinrichs, 2017). Therefore, appropriate protection of the protein against such stresses, during both manufacturing and storage, is necessary.

Sugars are widely used for the stabilization of proteins during drying and subsequent storage. During the drying process, sugars enclose proteins in a glassy matrix yielding a so-called sugar glass. In literature, there are two leading theories on the mechanism of stabilization of sugar glasses: the vitrification and water replacement theory (Cicerone, Pikal, & Qian, 2015; Mensink et al., 2017). Upon vitrification in a glassy matrix, the molecular mobility of proteins is strongly reduced, which in turn reduces degradation reaction rates (Chang et al., 2005; Crowe et al., 1998). Next to this kinetic stabilization mechanism, the water replacement theory has been proposed. This theory describes protein stabilization based on thermodynamics. According to the water replacement theory, the hydrogen bonds between the water molecules in the hydration shell of the protein and the protein itself are (partially) replaced by hydrogen bonds with the sugar (Allison, Chang, Randolph, & Carpenter, 1999; Mensink et al., 2015). This replacement conserves the three-dimensional structure of the protein. It is presumed that these two mechanisms are not exclusive and they simultaneously contribute to the stabilization of proteins (Crowe et al., 1998; Mensink et al., 2017). For optimal stabilization according to both theories, a close contact of the sugar with the protein and a high physical stability of the carbohydrate matrix, *i.e.* a high glass transition temperature (T_g), are required.

Abbreviations: BSA, bovine serum albumin; DSC, differential scanning calorimetry; DVS, dynamic vapor sorption; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RH, relative humidity; T_g, glass transition temperature; T_g', glass transition temperature of the maximally freeze concentrated fraction

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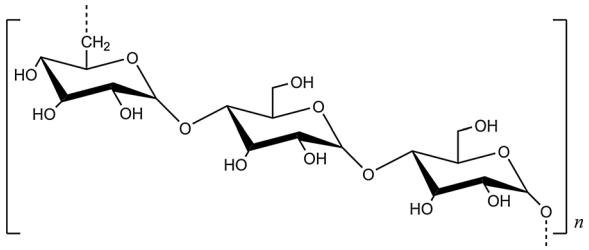

Fig. 1. Chemical structure of pullulan.

Numerous types of sugars have been explored as protein stabilizers (Chang et al., 2005; Hinrichs, Prinsen, & Frijlink, 2001; Tonnis et al., 2015). For various reasons outlined below, we hypothesize that the polysaccharide pullulan could be an attractive alternative. Pullulan is a linear and neutral exopolysaccharide produced by the yeast *Aureobasidium pullulans* consisting of repeating α-1,4 linked maltotriose units, which are connected by α-1,6 linkages (Fig. 1). Due to limited molecular flexibility rising from the latter connection, the conformation is believed to be random-coiled (Shingel, 2004). The molecular weight of the chains can vary between 45 and 2000 kDa (Cheng, Demirci, & Catchmark, 2011).

Over the last decades, pullulan has found various applications in the food and pharmaceutical industries. It is a tasteless, odorless, non-toxic and edible powder, and is generally regarded as safe. Pullulan readily dissolves in water and the resulting solutions are not affected by heat and pH changes (Prajapati, Jani, & Khanda, 2013). Therefore, pullulan solutions have for example been applied as low-viscosity fillers in blood plasma substitute solutions (Cheng et al., 2011; Prajapati et al., 2013). Furthermore, pullulan and pullulan derivatives have been applied in various drug delivery systems such as orodispersible films (Garsuch, 2009; Vuddanda et al., 2017), nanoparticles for protein delivery (Dionisio et al., 2013), and targeted delivery systems to the liver (Xi et al., 1996).

Although pullulan has many potential applications in drug delivery, it has never been thoroughly investigated as a sugar glass for protein stabilization. Yet, pullulan's high T_g and the absence of reducing groups would make it potentially an excellent candidate for protein stabilization. In fact, pullulan has been applied to enhance the stability of protein assay components by casting tablets in an exploratory study (Jahanshahi-Anbuhi et al., 2016). However, despite their high T_g, polysaccharides often show less efficient protein stabilization than the stabilization acquired by disaccharides, which have lower T_gs. Tonnis et al. (2015) hypothesized that polysaccharides are too bulky to form a compact coating around the protein. Furthermore, they also found that the addition of a disaccharide to a polysaccharide can improve the storage stability. This way, both a close contact between sugar and protein and a high physical stability are ensured.

Considering the above, we hypothesize that the addition of pullulan with its exceptionally high T_g to disaccharide glasses enhances the protein stabilizing properties, particularly at high humidity. Trehalose was selected as disaccharide as it has been well recognized as the gold standard for protein stabilization (Balcão & Vila, 2014; Manning et al., 2010). Firstly, physicochemical properties of pullulan and blends with a wide range of pullulan/trehalose ratios were assessed, including their behavior at conditions up to 90% relative humidity. Secondly, the stability of a model protein (β-galactosidase) in pullulan and pullulan/trehalose blends was tested at different temperature and humidity conditions. The applied high humidity conditions were chosen to demonstrate the specific beneficial effects of polysaccharides, as opposed to the low relative humidity conditions that are usually applied in storage stability studies with disaccharide stabilized proteins.

2. Materials and methods

2.1. Materials

Pullulan (average molecular weight 200–300 kDa, < 10% mono-, di- and oligosaccharides) was a kind gift of Hayashibara (Okayama, Japan). Trehalose was obtained from Cargill (Amsterdam, The Netherlands). β-galactosidase was purchased from Sorachim (Lausanne, Switzerland). Bovine serum albumin (BSA), disodium hydrogen phosphate dodecahydrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), lithium chloride, magnesium chloride hexahydrate, *ortho*-nitrophenyl-β-galactoside, monosodium phosphate dehydrate and potassium acetate were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Ammonium chloride was obtained from Spruyt-Hillen (IJsselstein, The Netherlands). Barium chloride was obtained from Merck (Darmstadt, Germany). Sodium bromide was purchased from Fagron (Capelle aan den IJssel, The Netherlands). Sodium nitrite was purchased from Boom (Meppel, The Netherlands). Potassium carbonate was obtained from Acros Organics (Geel, Belgium).

All solutions were prepared using Millipore water. The 0.1 M phosphate buffer used in this study consisted of 75 mM disodium hydrogen phosphate dodecahydrate and 25 mM monosodium phosphate dihydrate; pH was adjusted to 7.3 using 1 M NaOH. The pH of 2 mM HEPES buffer used in this study was set to 7.4.

2.2. Preparation of powder formulations

Pullulan and trehalose were accurately weighed and dissolved separately in water (dynamic vapor sorption and differential scanning calorimetry measurements) or HEPES buffer (all other samples). Next, the solutions were mixed in the following weight ratios of pullulan/trehalose: 1/0; 5/1; 2/1; 1/1; 1/2; 1/5 and 0/1 (final sample concentration of 50 mg/mL). For the storage stability experiments, β-galactosidase dissolved in HEPES buffer was added to each combination in a 1:249 protein:sugar weight ratio (final sample concentration of 50 mg/mL). As a negative control, a solution of β-galactosidase in HEPES buffer without any sugar was used. The closed vial storage stability samples were prepared in 4 mL vials, type I glass, Fiolax clear (Schott, Müllheim, Germany), with corresponding rubber stoppers (Salm & Kipp, Breukelen, The Netherlands). All other samples were prepared in 2 mL polypropylene vials. The vials were then immersed in liquid nitrogen to freeze the solutions and placed in a Christ-Epsilon 2-4 freeze-dryer (Salm & Kipp, Breukelen, The Netherlands) on a precooled shelf (–50 °C). Next, the samples were freeze-dried at a shelf temperature of −35 °C and a pressure of 0.220 mbar for 24 h. Secondary drying was performed by decreasing pressure to 0.050 mbar and increasing shelf temperature to 25 °C during 24 h. After freeze-drying, the 4 mL vials were closed under dry nitrogen gas to ensure 0% RH. Samples were stored at −20 °C until further analysis or subjection to a storage stability study.

2.3. Dynamic Vapor Sorption (DVS)

The water sorption isotherms of freeze-dried samples of all pullulan/trehalose compositions were determined using a DVS-1000 gravimetric sorption analyzer (Surface Measurement Systems Limited, London, UK). Samples had an initial mass of about 10 mg and were analyzed at ambient pressure and 25 °C. The water uptake by the sugar blends was measured from 0% to 90% RH in 10% increments. The RH was increased after equilibrium was achieved (change in mass < 0.5 μg within 10 min).