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Inulin glasses for the stabilization of therapeutic proteins

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Abstract

Sugar glasses are widely used to stabilize proteins during drying and subsequent storage. To act successfully as a protectant, the sugars should have a high glass transition temperature (Tg), a poor hygroscopicity, a low crystallization rate, and contain no reducing groups. When freeze drying is envisaged as method of drying, a relatively high Tg of the freeze concentrated fraction (Tg′) is preferrable. In this study, whether inulins meet these requirements was investigated. Inulins of various degrees of polymerisation (DP) were evaluated. Trehalose glass was used as a positive control. It was found that the Tg and the Tg′ of inulins with a number/weight average DP (DP_n/DP_w) higher than 5.5/6.0 were higher than those of trehalose glass. Furthermore, inulin glasses showed a similar hygroscopicity to that of trehalose glass but crystallized less rapidly. Less than 6% of the sugar units of inulins with a DP_n/DP_w higher than 5.5/6.0 contained reducing groups. Trehalose contained no reducing groups. Freeze drying of an alkaline phosphatase solution without protectant induced an almost complete loss of the activity of the protein. In contrast, when inulins with a DP_n/DP_w higher than 5.5/6.0 or trehalose were used as stabilizer, the activity was fully maintained, also after subsequent storage for 4 weeks at 20°C and 0, 45, or 60% RH, respectively. The stabilizing capacities of inulin with a lower DP and glucose were substantially less pronounced. After storage at 60°C for 6 days, the activity of freeze dried samples containing inulins with a DP_n/DP_w higher than 5.5/6.0 was still about 50% whereas the activity of samples containing inulin with a lower DP, glucose, or trehalose was completely lost. It is concluded that inulins with a DP_n/DP_w higher than 5.5/6.0 meet the physico–chemical characteristics to successfully act as protectants for proteins. The stabilizing potential of these inulins was clearly shown using alkaline phosphatase as a model protein.

Keywords: Inulin; Stabilization; Sugar glasses; Therapeutic proteins

1. Introduction

As a result of recent developments in the areas of molecular biology and biotechnology, peptides and proteins will become increasingly important as drug substances for a large variety of diseases and disorders. Inherent to the production process,

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proteins and peptides are obtained in aqueous solutions. However, dissolved in water, many proteins are unstable, limiting their shelf-life (Carpenter et al., 1997; Randolph, 1997; Shamblin et al., 1998a).

These problems may be solved when the protein is obtained in the dry state (Carpenter et al., 1997; Randolph, 1997; Shamblin et al., 1998a). However, during drying, e.g. freeze drying, evaporative drying or spray drying, the protein is subjected to harsh conditions through which its activity can be severely reduced. Therefore a protective agent is required to prevent the deleterious effects of drying.

From the literature it is well known that sugars can protect proteins during drying and subsequent storage (Slade and Levine, 1988, 1991; Slade et al., 1993; Slade and Levine, 1995; Cardona et al., 1997; Carpenter et al., 1997; Randolph, 1997; Schebor et al., 1997). The protective action is believed to be established according to the following mechanism: during drying, the water molecules surrounding the proteins molecules are gradually replaced by sugar molecules. Being a polyol, the sugar molecules form multiple external hydrogen bonds with the protein by which the structural integrity of the protein is maintained. Because the sugar molecules are in the amorphous state, a glass is eventually formed, provided that the temperature is below the Tg.

It is essential that during drying no crystallization of the sugar occurs. Crystallization is accompanied by phase separation through which the interaction of the sugar molecules with the protein and thereby the protection is lost (Izutsu et al., 1994; Luckel et al., 1997; Mazzobre et al., 1997b; Randolph, 1997; Rossi et al., 1997; Schebor et al., 1997; Terebiznik et al., 1997; Costantino et al., 1998). Therefore, the crystallization rate of the sugar used should be low.

For various reasons, it is essential that the sugar remains in the glassy state during handling and storage (Tg above room temperature). The molecular mobility in a glass is extremely low (Slade and Levine, 1988, 1995; Mazzobre et al., 1997a). Being immobilized in the glass, the protein is also protected against degenerating effects after the drying process. Furthermore, because of the restricted molecular mobility no crystallization of the sugar occurs within longer periods of time (years). In contrast, at temperatures above the Tg, the mobility increases to such an extent that the protection is (partially) lost (Slade and Levine, 1991, 1995; Duddu and Monte, 1997). The increased molecular mobility can also induce rapid crystallization of the sugar causing degradation as mentioned above. Finally, above the Tg, collapse of the material occurs which may result in handling problems (Luckel et al., 1997).

The Tg of a sugar glass depends on the nature of the sugar and on the water content (Slade and Levine, 1988, 1991, 1995). Water acts as a plasticizer and uptake of water by the sugar results in a strong decrease of the Tg. Consequently, it is highly desirable that the Tg of the glass is high and its hygroscopicity is low.

When freeze drying is chosen as the method of drying, it is preferrable that Tg′ is relatively high, because the sample temperature should remain below the Tg′ (Tzannis and Prestrelski, 1999). When the sample temperature is above Tg′, the freeze concentrated fraction is in the liquid or rubbery state and the molecular mobility is relatively high. Because the protein concentration in the freeze concentrated fraction is very high, the degradation rate will be increased when compared to the starting solution. Furthermore, the sugar concentration is very high and crystallization can easily occur with concomitant deteriorating effects to the protein. Furthermore freeze drying below the Tg′ results in a porous cake, while a collapsed cake is obtained above the Tg′. A porous cake is preferred because it can be reconstituted or processed more easily.

The absence of reducing groups is another requirement to be met by the applied sugar. Reducing groups can react with the amine groups of the protein to form a Schiff's base. This reaction is the first of a cascade of reactions also known as the Maillard reaction (Colaco et al., 1994; O'Brien, 1996; Carpenter et al., 1997). The Maillard reaction can severely affect the activity of the protein.

In this study, whether or not inulins meet the requirements mentioned above is investigated. Inulins were selected because they are nontoxic, already clinically applied, and a monograph in the USP exists. Furthermore, inulins of various molecular weights are available. By changing the molecular weight, the physico–chemical characteristics can be adjusted.

The stabilizing effects of inulins on a model protein, alkaline phosphatase were studied. Solutions containing inulin and alkaline phosphatase were freeze dried and subsequently stored under various conditions. After reconstitution, the activity of alkaline phosphatase was measured. Trehalose and glucose were used as positive and negative controls, respectively.

2. Materials and methods

2.1. Materials

Three different inulins, inulin SC 95, inulin RS, and inulin EXL 608, were a generous gift from Sensus, Roosendaal, The Netherlands. Alkaline phosphatase from bovine intestinal mucosa (10–30 DEA units per mg) was purchased from Sigma. All other chemicals were of reagent or analytical grade and purchased from commercial suppliers.

2.2. Methods

2.2.1. Degree of polymerisation of the different inulins

The DP_n and DP_w of the three inulins were determined by means of anion exchange HPLC as described by Timmerman et al. (1994). Briefly, separation was performed with a DIONEX system equipped with a Pulsed Electrochemical Detector using a CarboPac A1 column (4 × 250 mm) and a CarboPac A pre-column (4 × 50 mm). Samples were eluted with a mixture of solutions of sodium hydroxide and sodium acetate in water of which the ratio changed from 0.10:0.025 mol/l to 0.10:0.40 mol/l during a 60 min linear gradient. The system was calibrated using solutions of oligosaccharides of known concentrations and known molecular weights.

2.2.2. Determination of the number of reducing groups

The number of reducing groups was determined by means of the Sumner-assay according to the following procedure (Franssen et al., 1997). A solution of 20 g NaK–tartrate tetrahydrate, 1 g dinitrosalicylic acid, 1 g NaOH, and 200 mg phenol in 100 ml water was prepared. To 1.5 ml of this solution, 1.0 ml of an aqueous solution containing the sugar to be analysed was added. Subsequently, 100 μl of a freshly prepared solution of 0.24 M of Na_2SO_3 in water was added to this mixture. The resulting mixture was vortexed and then placed in a waterbath of 95°C. After 15 min, the samples were removed from the waterbath and allowed to cool to room temperature. The extinction of the samples was measured at 620 nm. The calibration curve was made using aqueous solutions with a glucose concentration of 0.10–1.00 mg/ml.

2.2.3. Freeze drying

Freeze drying was performed using a Christ model Alpha 2–4 lyophilizer (Salm en Kipp, Breukelen, The Netherlands). In a typical experiment, 20 ml glass vials were charged with 2 ml aqueous solutions. The solutions were froozen in liquid nitrogen and subsequently lyophilized at shelf temperature of −30°C, a condensor temperature of −53°C, and a pressure of 0.220 mBar for 18 h. Then, the shelf temperature and pressure were gradually raised to 20°C and 0.520 mBar, respectively during 6 h. Subsequently, the freeze drying process was continued for another 20 h under these conditions.

Sugar glasses without protein were prepared from solutions of 22.5 mg sugar per ml of water. Freeze dried alkaline phosphatase was prepared from a solution of 2.5 mg alkaline phosphatase per ml of aqueous 0.05 M Ammediol (2-amino-2-methyl-1, 3-propanediol), pH 9.8. Sugar glasses containing 10 wt.% of alkaline phosphatase were prepared from solutions of 22.5 mg sugar and 2.5 mg alkaline phosphatase per ml of aqueous 0.05 M Ammediol, pH 9.8.

2.2.4. Spray drying

Spray drying was performed using a Büchi 190 mini spray dryer (Büchi, Flawil, Switserland). Typical operating conditions were according to the following settings: air inlet temperature: 130°C, drying air flow 600 l/h, aspirator flow setting: 10, and pump control setting: 2.5. Sugar glasses were prepared from 10 wt.% solutions of sugars in water. The yield was 30–70%.

2.2.5. Alkaline phosphatase activity

The effect of lyophilization with and without protectant and subsequent storage under various conditions on the activity of alkaline phosphate was determined as described by Poelstra et al. (Poelstra et al., 1997b). The sample was reconstituted with water. To 50 μl of the sample, 905 μl of 0.05 M Ammediol, pH 9.8 and 20 μl of 100 mM $MgCl_2$ were added. Subsequently 50 μl of freshly prepared aqueous 10 mg/ml phosphatase substrate (*p*-nitrophenyl phosphate) solution was added. The mixture was vortexed and immediately incubated at 37°C. After 30 min, the reaction was quenched by adding 5.0 ml of 0.1 N NaOH and the extinction of the resulting mixture was measured at 405 nm. A calibration curve was established using freshly prepared solutions of alkaline phosphate in 0.05 M Ammediol, pH 9.8 of known concentrations.

2.2.6. Differential scanning calorimetry (DSC)

Calorimetric measurements were conducted with a DSC 2920 differential scanning calorimeter (TA instruments, Gent, Belgium). The glass transition temperatures (Tg) of dry as well as humidified sugar glasses were measured. The sugar glasses were prepared by freeze drying or spray drying. Sugar glasses were humidified at 20°C by transferring them into evacuated desiccators containing saturated aqueous solutions of CH_3COOK (relative humidity (RH) = 22%), $MgCl_2.6H_2O$ (RH = 33%), or $Na_2Cr_2O_72H_2O$ (RH = 52%) or by transferring them in climate chambers conditioned at 45 or 60% RH. The Tg of these samples were determined after 1–3 weeks of equilibration. The Tg was determined by either conventional DSC or modulated DSC (MDSC). To determine the Tg of humidified sugar glasses, the sample was cooled to −20°C and then heated to 200°C with rate of 20°C/min (DSC) or 2°C/min (MDSC). With MDSC, a modulation amplitude of ±0.318°C every 60 s was used. The Tg of the dry sugar glasses was determined by means of the same procedure except that the samples were preheated for 15–30 min at 90°C. During measurement, the sample cell was purged with nitrogen at a flow rate of 35 ml/min. The midpoint of the deflection in the heat flow versus temperature curve (DSC) or reversing heat flow versus temperature curve (MDSC) was taken as the Tg. Control experiments showed no significant differences between the Tg measured by DSC or by MDSC. No proper Tg of glucose glasses could be measured. This was due to severe handling problems caused by the extreme stickiness of the material even after short exposure to ambient atmosphere. The large variation of literature values (Slade and Levine, 1991; Wolkers et al., 1998) may reflect these problems (Table 1).

The Tg′ of aqueous sugar solutions of different concentrations was also determined, measured by means of conventional DSC only. Solutions were cooled to −70°C with a cooling rate of 10°C/min. Subsequently, the samples were heated to 40°C with a rate of 20°C/min. During these measurements, the sample cell was purged with helium at a flow rate of 35 ml/min. The midpoint of the deflection in the heat flow versus temperature curve was taken as the Tg′.

2.2.7. Dynamic vapour sorption

Water sorption isotherms for sugar glasses were measured at ambient pressures and 25°C using a gravimetric sorption analyser (DVS-1000 Water Sorption Instrument, Surface Measurement Systems Limited, London, UK). The uptake of water by sugar glasses was measured from 0 to 90% RH with steps of 10% RH. The initial sample weight was about 10 mg. It was assumed that equilibrium was reached when the change of weight was less than 0.9 μg during a 10 min period. The sugar glasses were prepared by spray drying. Due to the same handling problems as described before, glucose glass was omitted from these measurements.

3. Results

3.1. Degree of polymerisation

The DP_n and DP_w increased in the order inulin SC 95 < inulin RS < inulin EXL 608 (Table 1). The polydispersity of the samples was relatively small and increased in the order inulin SC 95 ($DP_w/DP_n = 1.09$) < inulin EXL 608 ($DP_w/DP_n = 1.13$) < inulin RS ($DP_w/DP_n = 1.36$).

3.2. Reducing groups

The number of reducing groups was determined by means of the Sumner assay. Due to the specific linkage of the two glucose units, trehalose contains no reducing groups (Table 1). Inulins consist of a linear β-D-(2→1) linked fructose oligomer ending with a α-D-(1→2) glucopyranose ring. Therefore, theoretically inulins should contain no reducing groups as well. However, the Sumner assay clearly showed the presence of reducing groups in the three different inulins, especially in the low molecular weight inulin, SC 95 (Table 1). The very high content of reducing groups in inulin SC 95 can be ascribed to the presence of monosaccharides and inulin species of which the glucose endgroup is cleaved. The monosaccharides are most likely glucose and fructose. Fructose is a nonreducing sugar. However, during the Sumner assay, the sugar is subjected to a high temperature by which fructose can be easily converted into glucose (Lobry de Bruyn van Ekenstein rearrangement). Indeed in control experiments, it was found that fructose displayed one reducing group per molecule in the assay (data not shown). Therefore, the measured amount of reducing groups is probably overestimated.

The few reducing groups in inulin RS and EXL 608 are most likely predominantly caused by the presence of inulin species of which the glucose endgroup is cleaved although the presence of monosaccharides may have contributed too.

3.3. Glass transition temperatures

As found by others (Taylor and Zografi, 1998; Shamblin et al., 1998b), the Tg of trehalose glass was 121.6°C. When exposed to air of increasing RH at 20°C, the Tg sharply dropped to −3.2°C after storage at 60% RH (Fig. 1). The decrease in Tg reflects the plastizing effect of water. Furthermore, when exposed to air of 52% RH, trehalose

Table 1
Physico–chemical characteristics and stabilizing effect on alkaline phosphatase of inulins, trehalose and glucose

	DP_n/DP_w[a]	Tg (°C)[b]	Tg′ (°C)[b,c]	% Sugar units containing reducing groups[d]	Activity alkaline phosphatase after freeze drying (%)[e]
Inulin SC 95	5.5/6.0	102.0 ± 3.0	−26.2 ± 0.1	53.6 ± 2.4	110.0 ± 2.4
Inulin RS	14.2/19.4	139.7 ± 1.7	−19.2 ± 0.1	2.1 ± 0.1	94.4 ± 10.6
Inulin EXL 608	23.0/26.2	154.4 ± 1.8	−17.0 ± 0.3	5.2 ± 0.3	95.0 ± 2.5
Trehalose	–	121.6 ± 0.7	−29.2 ± 0.3	0.14 ± 0.04	95.9 ± 11.5
Glucose	–	21–39[f]	−39.3 ± 0.1	100[g]	110.3 ± 10.6
No protectant	–	–	–	–	5.4 ± 2.1

[a] DP_n: number average degree of polymerisation; DP_w: weight average degree of polymerisation.
[b] Data are the mean of two to four independent experiments; ± SD.
[c] Tg′ of 10 wt.% solutions.
[d] Data are the mean of three independent experiments; ± SD.
[e] Solutions of 22.5 mg sugar and 2.5 mg alkaline phosphatase per ml of aqueous 0.05 M Ammediol, pH 9.8 were freeze dried. After reconstitution the activity of the protein in the sample was measured. Data are the mean of two independent experiments; ± SD.
[f] Literature values (Slade and Levine, 1991; Wolkers et al., 1998).
[g] Theoretical value.

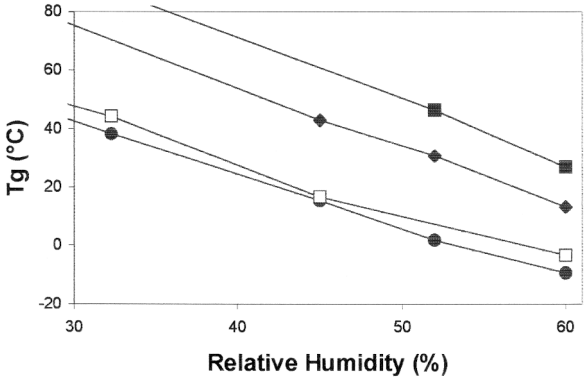

Fig. 1. Tg of sugar glasses as a function of the RH to which they were exposed at 20°C. –■– inulin EXL 608, –◆– inulin RS, –●– inulin SC 95, and –□– trehalose. Data are the mean of three to five experiments (SD was less than 3°C).

glass rapidly changed into a very viscous liquid. Subsequently, this viscous liquid changed into a hard brittle material within a few days indicating crystallization. These observation are in agreement with literature (Crowe et al., 1996; Cardona et al., 1997; Iglesias et al., 1997; Rossi et al., 1997; Terebiznik et al., 1997).

The Tg of the three different inulins clearly increased with DP (Table 1). The effect of molecular weight on the Tg is a well known phenomenon in oligosaccharide chemistry and follows the Fox–Flory equation: $Tg = Tg_x - C/MW$ where Tg_x is the Tg at infinite molecular weight, C is a constant, and MW is the molecular weight of the sugar (Her and Nail, 1994; Cardona et al., 1997). The increase of the Tg with increasing molecular weight can be ascribed to a decrease in chain ends which largely contribute to the free volume (Slade and Levine, 1988, 1991). The Tg of inulin SC 95 as a function of the RH strongly resembled that of trehalose (Fig. 1). However, after exposure to air of 52% RH, no crystallization was observed even after 3 weeks of storage. Over the whole range of humidities tested, inulin RS possessed a substantial higher Tg and inulin EXL 608 an even higher Tg (Fig. 1). The plots of Tg against the RH of all the materials tested were more or less parallel indicating that all these materials show a similar hygroscopicity.

The Tg′ was measured by rapidly freezing solutions of different concentrations and subsequent heating of the samples in a DSC instrument. Although theoretically there should be no dependence on the concentration of the starting solution, the Tg′ slightly increased with increasing concentration in all cases (Fig. 2). This dependence has been found before but could not be explained adequately (Her and Nail, 1994; Jang et al., 1995). The Tg′ of glucose and trehalose were about -40 and -30°C, respectively, which is in agreement with literature values (Franks, 1990; Slade and Levine, 1991; Schenz et al., 1993). The Tg′ of inulin SC 95 was about the same as that for trehalose. The other inulins had a substantial higher Tg′ (Fig. 2). As for the Tg, the Tg′ of

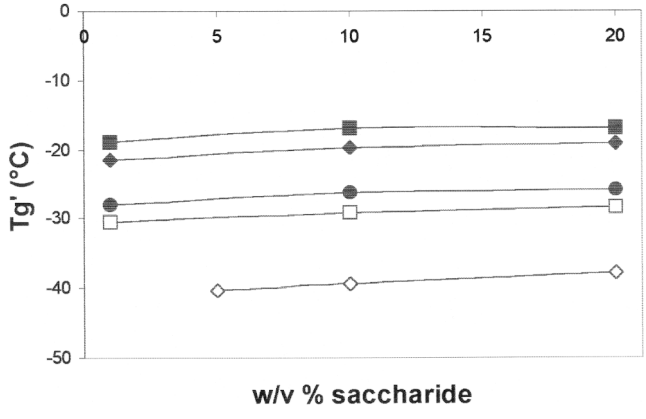

Fig. 2. Tg′ of solutions as a function of the sugar concentration. –■– inulin EXL 608, –◆– inulin RS, –●– inulin SC 95, –□– trehalose, and –◇– glucose. Data are the mean of three to five experiments (SD was less than 0.3°C).

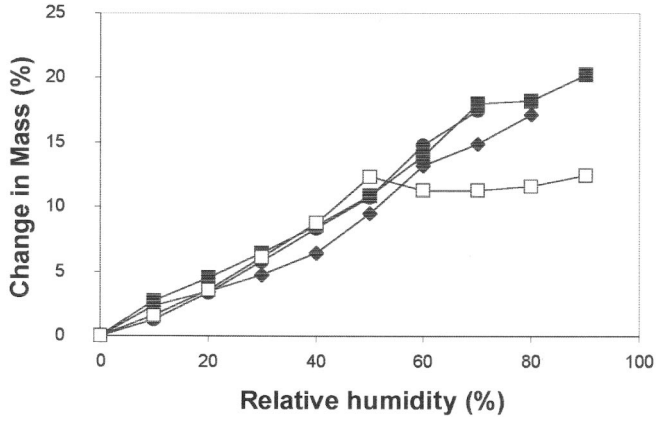

Fig. 3. Water sorption isotherms of sugar glasses at 25°C. –■– inulin EXL 608, –◆– inulin RS, –●– inulin SC 95, and –□– trehalose.

oligomer saccharides follow the Fox–Flory equation (Ablett et al., 1993; Schenz et al., 1993; Her and Nail, 1994; Jang et al., 1995; Slade and Levine, 1995). As a result, the Tg′ increased in the order inulin SC 95 < inulin RS < inulin EXL 608. These results indicate that the sugar/water weight ratios in the freeze concentrated fractions showed no large variations.

3.4. Hygroscopicity of sugar glasses

The moisture uptake of sugar glasses exposed to air of relative humidities ranging from 0 to 90% at 25°C was measured using a gravimetric sorption analyser. It was found that up to 50% RH, the uptake of water by trehalose glass increased linearly with the RH (Fig. 3). At higher RH's, no further uptake was measured. As shown by DSC measurements, at 50% RH and higher, the Tg of trehalose drops to below 25°C. Therefore, at these RH's, trehalose became rubbery and crystallization occurs. Above 50% RH, a weight gain of about 11.5% was measured which closely corresponds to a trehalose/water mol ratio of 1/2. Since it is known that trehalose crystallizes as a dihydrate (Crowe et al., 1996; Ding et al., 1996; Iglesias et al., 1997; Rossi et al., 1997; Terebiznik et al., 1997), these results strongly indicate that complete crystallization of the sample had occurred. Apparently, crystallization of trehalose under these conditions proceeds rapidly since it was completed within a few hours. Similar results were found by Iglesias et al. (1997). The three different inulins showed a linear relationship up to 90% RH when the uptake of water was plotted against the RH. This result indicates that during the experiment no crystallization of inulin occurred although all samples passed the Tg at some point (Fig. 3). Apparently, inulins crystallize less easily than trehalose. All materials tested showed a similar uptake of water up to 50% RH. This indicates that all these glassy materials are all about equally hygroscopic which is in agreement with the results of the DSC experiments.

3.5. Stabilizing effect of sugars

To evaluate the stabilizing effect of glucose, trehalose and the three different inulins, aqueous solutions of alkaline phosphatase without protectant and solutions containing alkaline phosphatase and one of the sugars in a weight ratio of 1/9 were freeze dried. After freeze drying, the samples were stored under various conditions after which the activity of the protein was measured. It was found that freeze drying of an aqueous alkaline phosphatase solutions without protectant had a deteriorating effect on the protein: only $5.4 \pm 2.1\%$ of the original activity remained (Table 1). In contrast, when either one of the inulins, trehalose, or glucose was added no significant loss of activity was observed (Table 1).

When stored at 20°C and 0, 45, or 60% RH no significant change of the activity occurred up to a storage time of 4 weeks, except for the samples containing inulin SC 95 and glucose stored at 45% and 60% RH (Fig. 4a–c). In the latter cases, the activity of the protein was substantially decreased after 4 weeks of storage. This decrease was more pronounced when the samples were stored at 60%

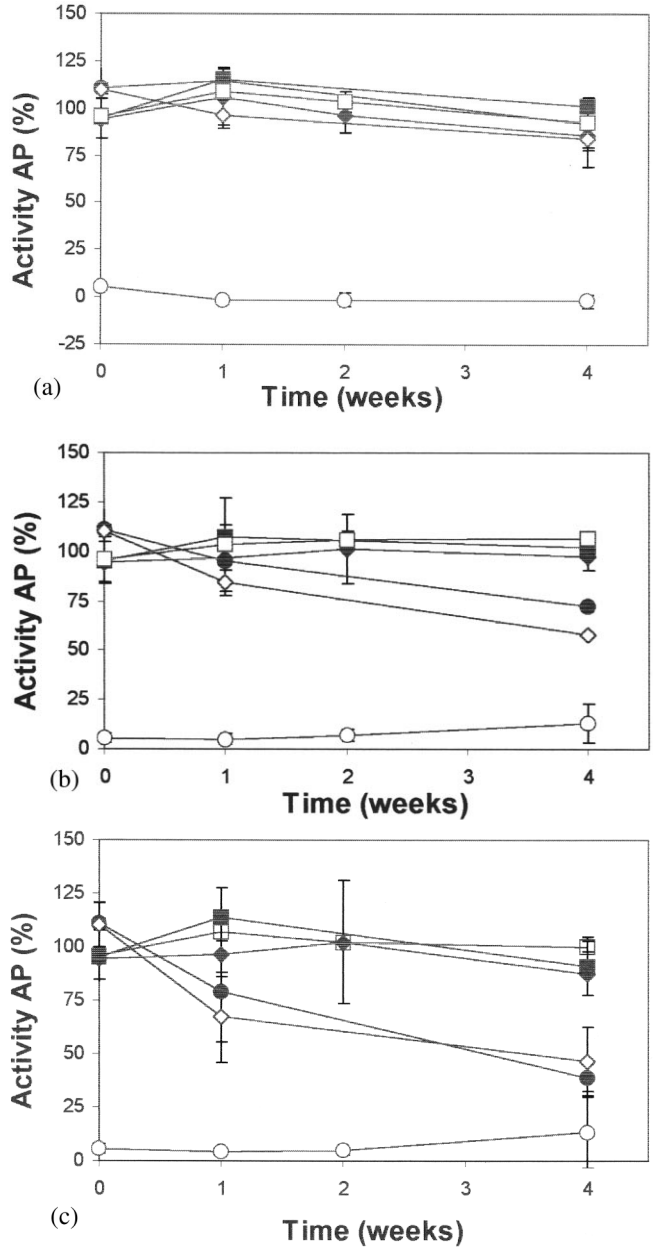

Fig. 4. Activity of alkaline phosphatase freeze dried in the presence of various sugars and stored at 20°C and 0% (a), 45% (b), and 60% RH (c) as a function of storage time. –■– inulin EXL 608, –◆– inulin RS, –●– inulin SC 95, –□– trehalose, –◇– glucose, and –○– no protectant. Data are the mean of two independent experiments; ± SD.

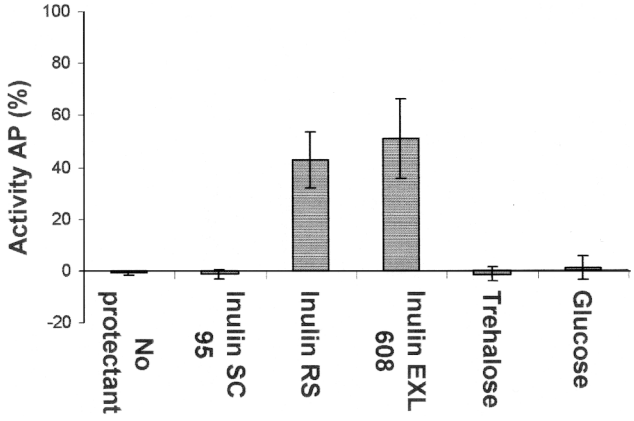

Fig. 5. Activity of alkaline phosphatase freeze dried in the presence of various sugars and stored at 60°C and 0% RH for 6 days. Data are the mean of two independent experiments; ± SD.

RH than at 45% RH. In both cases, the gradual loss of activity was accompanied by change of color from white to red/brown. No color formation was observed in the other samples. When stored at 60°C and 0% RH for 6 days, the samples containing inulin SC 95, trehalose, or glucose showed no significant activity anymore, while the activity of the samples containing inulin RS or inulin EXL 608 was still 42.8 ± 10.8% and 50.9 ± 15.3%, respectively, of the original value (Fig. 5). The samples containing glucose or inulin SC 95 became red/brown. The other samples remained white.

4. Discussion

The results of this study indicate that inulins with a DP_n/DP_w higher than 5.5/6.0 meet the physico–chemical requirements to succesfully act as a protectant for therapeutic proteins. The excellent protective potential of these inulins was confirmed by the stabilization of alkaline phosphatase during freeze drying and subsequent storage under various conditions.

Trehalose has been described in many publications as the sugar of choice to stabilize proteins (Colaco et al., 1992, 1994; Li et al., 1996; O'Brien, 1996; Schebor et al., 1996; Carpenter et al., 1997; Mazzobre et al., 1997b; Sun, 1999). The excellent protective properties of trehalose glass have been ascribed to the high Tg, poor hygroscopicity, absence of reducing groups, and low crystallization rate. The major advantage of inulins is their variable molecular weight by which the Tg can be adjusted. At zero moisture content, the shortest inulin used in this study, inulin SC 95, had a somewhat lower Tg than trehalose, the other two inulins having a substantial higher Tg (Table 1). Moisture sorption experiments revealed that the three inulins and trehalose glasses are about equally hygroscopic up to 50% RH (Fig. 3). As a consequence, after exposure to humidified air, the Tg of trehalose and inulin SC 95 are about the same. Both are substantially lower than those of inulin RS and EXL 608 (Fig. 1). This implies that inulin RS and inulin EXL 608 can be exposed to much higher RH's without passing the Tg than inulin SC 95 and trehalose. For example at 20°C, inulin RS and inulin EXL 608 can still be exposed to a RH of 55% and more than 60%, respectively while trehalose and inulin SC 95 can not be exposed to a RH of more than about 40%. Since an ambient atmosphere of around 40% RH is not uncommon, these data imply that special precautions have to be taken when trehalose and inulin SC 95 glasses have to be processed, e.g. into dosage forms like freeze dried injections, formulations for pulmonary delivery, or tablets for oral administration. For inulin RS and inulin EXL 608 these precautions are not necessary or to a lesser extent. The very low Tg of glucose glass makes this material unsuitable for processing.

Furthermore, it is possible that during storage, shipping, or processing, the Tg is accidentally passed for some time. Because of their low Tg, this is more likely to occur with trehalose and inulin SC 95 than with inulin RS and inulin EXL 608. When a protein is incorporated in the glass and full crystallization of the sugar occurs, the stabilization will be completely lost (Izutsu et al., 1994; Mazzobre et al., 1997b; Randolph, 1997; Rossi et al., 1997; Schebor et al., 1997; Terebiznik et al., 1997; Costantino et al., 1998). However, when the Tg is passed but crystallization will not occur, the stabilization will only be lost to a limited extent because of the increased molecular

mobility. However, most of the stabilizing effects is preserved because the interaction between sugar and protein remains intact (Cardona et al., 1997). Moisture sorption experiments showed that above the Tg, trehalose crystallizes at a much higher rate than the three inulins. Therefore, based on their slow crystallization rate, it is expected that above the Tg, inulins provide a prolonged protection.

The Tg′ of sugar glasses increased in the order: glucose < trehalose ≈ inulin SC 95 < inulin RS < inulin EXL 608 (Fig. 2). As a consequence, solutions containing inulin RS or inulin EXL 608 can be freeze dried at substantially higher sample temperatures than solutions containing inulin SC 95 or trehalose. This is highly advantageous because freeze drying at a higher sample temperature proceeds at a higher rate, which makes the drying process less expensive and more energy efficient.

As expected no significant amounts of reducing groups were found for trehalose. Unexpectedly, the short chain inulin clearly showed the presence of reducing groups. The high amount of reducing groups in inulin SC 95 is expected to substantially initiate the Maillard reaction which may severely reduce the activity of proteins (Colaco et al., 1994; Li et al., 1996; O'Brien, 1996; Carpenter et al., 1997). Inulin RS and inulin EXL 608 also contain reducing groups, however, at a much lower level than in inulin SC 95. Therefore, severe deterioration of the protein due to the Maillard reaction is less likely to occur.

Based on their physico–chemical characteristics, it was proposed that the two relatively high molecular weight inulins, inulin RS and inulin EXL 608, may successfully act as a protectant for therapeutic proteins during drying and subsequent storage. This hypothesis was tested using alkaline phosphatase. This particular protein was selected because it is currently in development for the treatment of septic shock (Poelstra et al., 1997a). Furthermore, alkaline phosphatase from intestinal bovine sources is relatively unstable (Ford and Allahiary, 1993; Ford and Dawson, 1993), in particular against temperature changes (McComb et al., 1979). Its instability was evident as freeze drying without protectant resulted in a dramatical decrease of the activity. Full protection was achieved by the application of all three inulins as well as by trehalose and glucose. This full protection was maintained for at least 4 weeks at 0% RH and 20°C even in the case of the negative control glucose (Fig. 4a). Apparently, alkaline phosphatase is quite stable under these extremely dry conditions and no distinction can be made between a poor and an effective protectant. Therefore, the samples were challenged more severely by raising the RH to 45 and 60%. Under these conditions only the two relatively high molecular weight inulins, RS and EXL 608, and trehalose were able to fully protect the protein against deterioration for at least 4 weeks. The activity of the samples containing the lower molecular weight inulin, SC 95, or glucose was substantially decreased after 4 weeks (Fig. 4b–c). Furthermore, these samples gradually became red/brown. Since these sugars contain high amounts of reducing groups, the results indicate that the Maillard reaction had occurred. Obviously, the substantial decrease of the activity can be ascribed to the low Tg of the used sugars in combination with the occurrence of the Maillard reaction. When stored at 60°C and 0% RH for 6 days, differences between the stabilizing capacities of the sugars were even more pronounced (Fig. 5). Not only the samples containing inulin SC 95 or glucose but also the sample which contained trehalose showed no significant activity anymore. In contrast, the higher molecular weight inulins, RS and EXL 608, were able to substantially protect alkaline phosphatase against degradation.

In the literature, inulin has been described as a poor protectant for proteins (Colaco et al., 1992, 1994). From these publications it is unclear which type of inulin was used. It is likely that an inulin of low DP and/or a high content of reducing groups was used since the results of the present study clearly show that these inulins are indeed poor protectants. However, inulins of relatively high DP and low content of reducing groups have shown to be excellent protectants.

In conclusion, the physio–chemical characteristics of inulins with a DP_n/DP_w higher than 5.5/6.0 make these oligosaccharides highly suitable to stabilize proteins during drying and subsequent storage.

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