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## Analytical techniques and formulation strategies for the therapeutic protein alkaline phosphatase

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## Chapter 6

### Investigations into the stabilization of drugs by sugar glasses: Tablets prepared from stabilized alkaline phosphatase

H.J.C. Eriksson, W.L.J. Hinrichs, B. van Veen, G.W. Somsen, G.J. de Jong,  
H.W. Frijlink. *Int J Pharm*, 2002. **249**: p. 59-70.

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**Summary**

The aim of this study was to investigate the formulation of sugar glass stabilized alkaline phosphatase from bovine intestine (BIAP) into tablets. Two major subjects of tablet formulation were investigated. First, the compaction behaviour of the inulin sugar glass was investigated. Secondly, the effect of the compaction process on the physical stability of sugar glass stabilized BIAP was evaluated, comparing inulin and trehalose glass. The tableting properties of freeze-dried inulin without BIAP were studied first. Freeze-dried inulin conditioned at 20 °C/0% relative humidity (RH) or 20 °C/45% RH was compacted at various pressures. As expected, the yield pressure of the material conditioned at 0% RH was higher (68 MPa) than after conditioning at 45% RH (39 MPa). Tablets made of the material stored at 0% RH showed severe capping tendency, especially at high compaction pressures. In contrast, material conditioned at 45% RH gave tablets without any capping tendency and a friability of less than 1%.

Sugar glasses of BIAP and either inulin or trehalose were prepared by freeze-drying (BIAP/sugar 1/19 (w/w)). The material was subsequently compacted. Tablets and powders were stored at 60 °C/0% RH. The activity of the incorporated BIAP was measured at various time intervals. It was found that inulin was by far superior to trehalose as stabilizer of BIAP in tablets. The poor stabilizing capacities of trehalose after compaction are explained by crystallization of trehalose induced by the compaction process and moisture in the material.

The results clearly show that inulin is an excellent stabilizer for BIAP. The tableting properties are adequate, showing sufficient tablet strengths and low friability. Furthermore, the good (physical) stability of inulin glass with respect to exposure to high relative humidities makes it practical to work with.

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## 6.1 Introduction

Pharmaceutically active proteins have been applied for decades. However, their number was small until the 1980s, but since that time rapid developments in molecular biology resulted in a fast increase. Currently, the FDA has approved over 30 different recombinant DNA-derived proteins, e.g. erythropoietin, interferon alpha-2a/b, somatropin, and follitropin beta and many more are already in a far stage of development. This fast growth calls for the development of dosage forms that provide stability of the drug during manufacturing and subsequent storage and that also allow patient friendly administration.

Stabilization of proteins can be achieved by mixing the protein solution with a sugar after which the solution is lyophilized or spray-dried. If dried properly, the protein is incorporated in a matrix consisting of amorphous sugar in its glassy state. Among other things, stabilization is achieved because the mobility of the protein is strongly reduced. Several review articles that cover this subject have been published [1-3]. It is often claimed that trehalose is superior as stabilizer when compared to other sugars, like sucrose, maltose, raffinose or lactose [4-11]. However, recently it was shown that also inulin provides excellent stabilization of proteins during freeze-drying and subsequent storage [12].

Once in the dry state, it is possible to develop other than liquid dosage forms, such as tablets or powders for inhalation. Although there is a substantial amount of papers dealing with the stabilization of proteins by lyophilization or spray-drying, papers on the formulation of these drugs for oral administration are scarce. Nonetheless, quite recently a paper on the stabilization of an antibiotic freeze-dried with trehalose and subsequently compacted was presented [11]. However, no experimental details were mentioned. In a previous study spray-dried inulin was tested as excipient for direct compaction [13]. It was shown that this material has excellent tableting properties. In the study by Hinrichs *et al.* [12] the possibility to use inulin as a stabilizer of proteins was explored, while in the study by Eissens *et al.* [13] the compaction properties of spray-dried inulin without the presence of a protein were investigated.

The aim of this study was to investigate the formulation of sugar glass stabilized alkaline phosphatase from bovine intestine (BIAP) into tablets. Two major subjects of tablet formulation were investigated. First, the compaction behaviour of the inulin sugar glass was evaluated.

Secondly, the effect of the compaction process on the physical stability of sugar glass stabilized BIAP was investigated, comparing inulin and trehalose glasses.

Alkaline phosphatase (AP) is a thermolabile enzyme [14, 15] that currently is investigated within the University of Groningen as a potential treatment of sepsis, which is caused by endotoxins produced by Gram-negative bacteria. In the case of sepsis the permeability across the intestinal wall increases, which might allow endotoxins to enter the blood stream. The AP can detoxify these endotoxins by removal of their phosphate groups. Since a local effect in the intestinal lumen is desired delivery of AP via the oral route is preferred.

## 6.2 Material and methods

### *Material*

Inulin with a number/weight average degree of polymerization ( $DP_n/DP_w$ ) of 23/26 was a gift from Sensus (Rosendaal, The Netherlands), D-(+)-trehalose and alkaline phosphatase from bovine intestine mucosa (BIAP) were purchased from Sigma (St Louis, MO, USA), para-nitrophenylphosphate and 2-amino-2-methyl-1,3-propanediol were purchased from Sigma-Aldrich (Steinheim, Germany), and  $MgCl_2$  was from Fluka (Buchs, Switzerland). NaOH and HCl were purchased from Merck (Darmstadt, Germany).

### *Freeze-drying*

Solutions of inulin (10% w/v in demineralized water), BIAP/inulin, BIAP/trehalose (both 1/19 w/w, 10% w/v in 0.05 M ammediol (pH 9.8)) and BIAP without sugar (0.25% w/v in 0.05 M ammediol (pH 9.8)) were rapidly frozen in liquid nitrogen. Freeze-drying was carried out in a Christ Alpha 1-4 freeze-dryer (Salm en Kipp, Breukelen, The Netherlands) as follows; 96 hours at a shelf temperature of  $-35\text{ }^\circ\text{C}$ , a condenser temperature of  $-53\text{ }^\circ\text{C}$ , and a pressure of 0.220 mbar followed by a stepwise increase during 6 h to  $20\text{ }^\circ\text{C}$  and 0.520 mbar, which then was maintained for another 20 hours. After freeze-drying, the samples were kept in a vacuum desiccator for at least four days.

*Compaction behaviour of freeze-dried inulin*

Before compaction freeze-dried inulin was gently ground and then equilibrated at either 20 °C/0% RH or 20 °C/45% RH. Tablets (round, flat, diameter 13 mm, weight 300 mg) were prepared with a compaction simulator (ESH, Brierley Hill, UK) at an average compaction speed of 3 mm/s. In the compaction chamber the temperature was 19 °C and the relative humidity was 60%. The compaction pressures varied between 7 and 210 MPa. Between each compaction the die was lubricated with magnesium stearate. The upper punch displacements were sine waves with different amplitudes in order to obtain different compaction pressures. The lower punch was stationary during compaction and the ejection time was always 10 s. The yield pressure of the inulin was calculated according to Heckel [16]. In short, the  $-\ln$  (porosity under pressure) is plotted against the compaction pressure and then the equation of the linear region is calculated. The yield pressure is retrieved from the reciprocal of the slope. After ejection the tablets were stored for at least 16 h at 20 °C and a relative humidity as before compaction. To determine the porosity of the tablets after relaxation their dimensions were measured with an electronic micrometer (Mitutoyo, Tokyo, Japan) and the tablets were weighed on an analytical balance (Mettler-Toledo, Greifensee, Germany). The density of the tablets ( $D_T$ ) was calculated and then the porosity was calculated as  $1 - D_T/D_t$ , where  $D_t$  = the true density of inulin glass (1.480 g/cm<sup>3</sup> for inulin at 45% RH and 1.534 g/cm<sup>3</sup> for inulin at 0% RH) [13]. The crushing strengths were measured with the compaction simulator as described previously [17, 18].

The friability of tablets made from material conditioned at 20 °C/45% RH was tested according to the European Pharmacopoeia [19].

*Production of tablets for stability testing*

Tablets for the stability testing of BIAP incorporated in inulin or trehalose glasses were produced using a hydraulic press (ESH, Brierley Hill, UK). The material containing inulin had been conditioned at 20 °C/45% RH, while the material containing trehalose had been conditioned at 20 °C/0% RH in order to prevent crystallization. The RH in the room where compaction took place was 70% and the temperature was 20 °C. The weighing of powders followed by compaction was performed as fast as possible (< 1 minute per tablet). A compaction pressure of 110 MPa was used for all the tablets. Immediately after compaction the tablets were

transferred to a vacuum desiccator. After 16 to 20 hours the enzymatic activity was measured. Tablets from each material were stored at 60 °C/0% RH or in the vacuum desiccator at room temperature. Uncompacted powders were stored similarly. The enzymatic activity of the BIAP was measured at different time intervals up to 3 months.

#### *Physical stability of freeze-dried trehalose and inulin*

Trehalose and inulin were freeze-dried from aqueous solutions that contained 10% w/v of the respective sugars as described above. After freeze-drying the materials were stored at 0% RH in a vacuum desiccator for at least 2 days. Trehalose was then stored at 0, 33 and 45% RH, respectively, while inulin was stored at 0 and 45% RH, respectively, all at room temperature. The freeze-dried trehalose and inulin powders and tablets prepared from these powders were also stored at 60 °C/0% RH and 60 °C/33% RH. The tablets were made after the materials had been stored for at least two weeks in their respective climates. The compaction process took place under a stream of dry nitrogen in order to achieve 0% RH and thus eliminate the influence of moisture present in the compaction chamber. The physical appearance of the powders was investigated before compaction. Furthermore, the thermal behaviour of the samples was evaluated in duplicate using differential scanning calorimetry (DSC) at a scanning rate of 20 °C/min in open aluminium pans using approximately 10 mg for each measurement. The instrument, a TA Instruments DSC 2920 (TA instruments, Ghent, Belgium), had been calibrated with indium and the instrument was also equipped with a cooling device that was supplied with a stream of nitrogen throughout the measurements.

#### *Enzymatic activity assay*

Just before the enzymatic activity assay the tablets were gently crushed to smaller pieces in a mortar. From each sample duplicates were weighed and each duplicate was assayed twice, according to a previously published method [12]. For each analysis a calibration curve in the range 0 to 40 µg/ml of BIAP was prepared from untreated BIAP, which was stored at -18 °C. All samples were dissolved and diluted in 0.05 M ammonium dihydrogen phosphate in water (pH 9.8) to yield a final concentration of BIAP of about 25 µg/ml. The enzymatic activity of the samples was determined by measuring the conversion of pNPP to its yellow product para-nitrophenol. For the assay 900 µl of a mixture of 97.8% v/v of 0.05 M ammonium dihydrogen phosphate (pH 9.8)

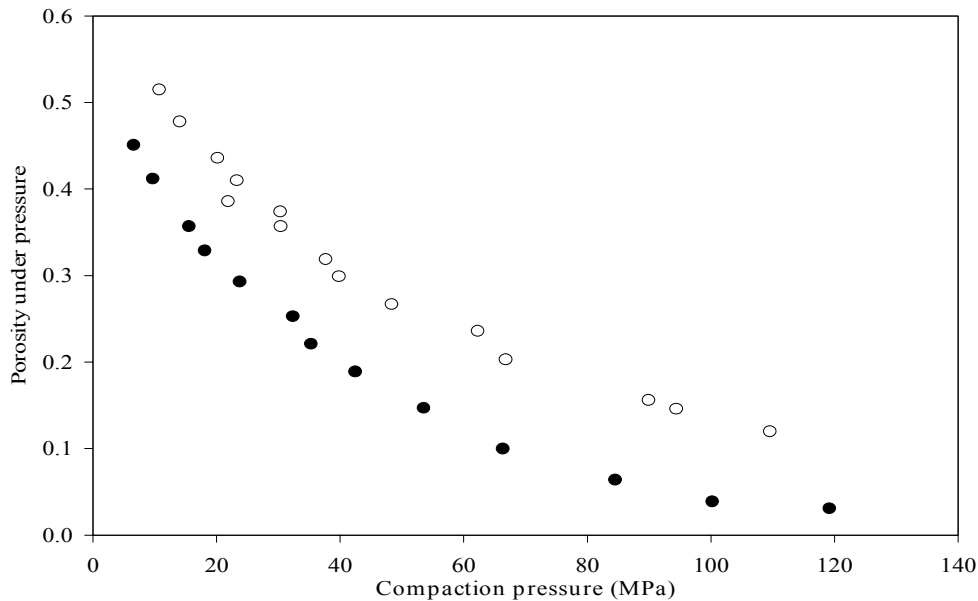
with 2.2% v/v of 100 mM MgCl<sub>2</sub> and 50 µl of sample were mixed with 50 µl 10 mg/ml pNPP in demineralized water. Immediately after the addition of the pNPP-solution the reaction mixtures were vortex mixed and then placed in a water bath (P.M. Tamson N.V., The Netherlands) set to 37 °C for 30 min. The reaction was quenched by adding 5.00 ml 0.1 M NaOH to the reaction solution. The absorbance at 405 nm of the samples was then measured using a Philips PU 8720 spectrophotometer (Philips, The Netherlands).

### 6.3 Results and Discussion

#### *Compaction behaviour and tablet properties of freeze-dried inulin*

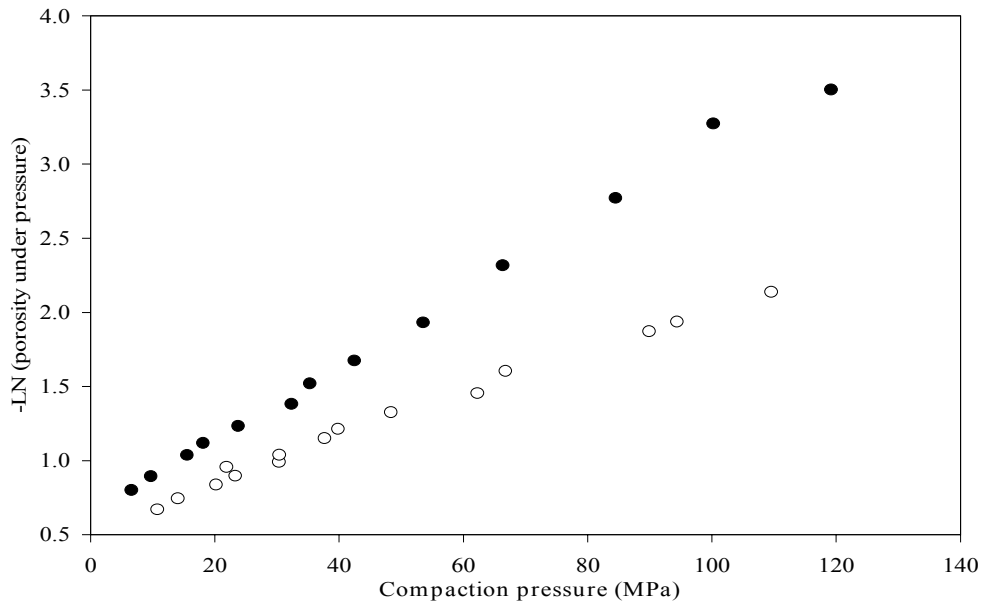
The compaction behaviour of freeze-dried inulin was studied using the powders conditioned at 20 °C/0% RH and 20 °C/45% RH. The dry material showed capping tendency at compaction pressures higher than 67 MPa. When the tablets were ejected from the die, this capping process was evident within a few seconds as an increase in tablet height was clearly visible and the tablets split. This behaviour is explained by the storage of elastic energy, which was released as a fracture in the tablet when the pressure was removed. In contrast, tablets made from the material stored at 45% RH showed no capping, and tablets with high tensile strengths could be prepared. This behaviour has also been found for amorphous lactose [20]. In Fig. 1 the densification of both powders are shown as the porosity under pressure. The porosity under pressure was higher for the dry material compared to the moist material showing a difference in densification behaviour of the powders.





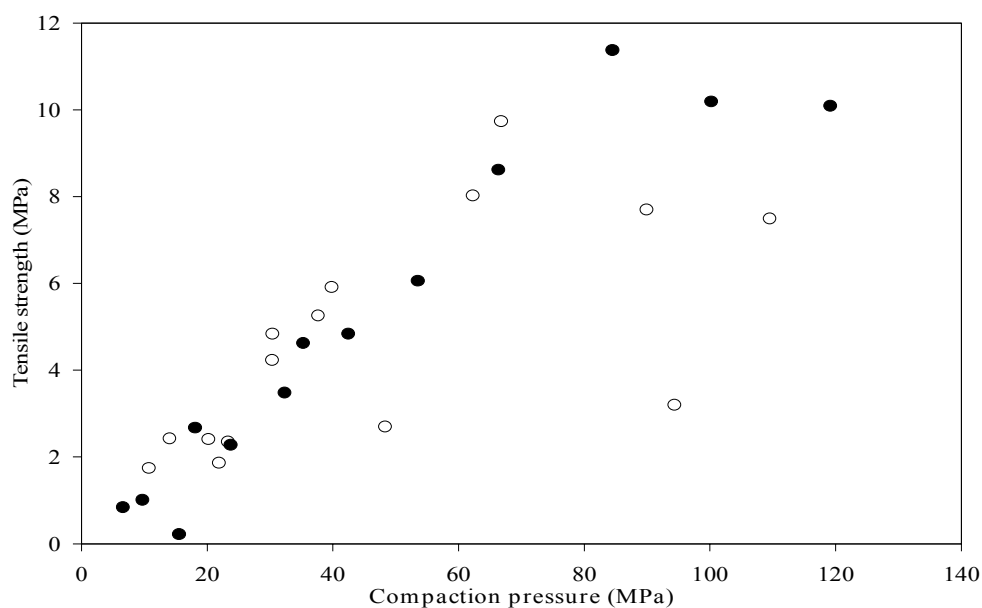
**Fig. 1** The porosity under pressure versus compaction pressure for freeze-dried inulin conditioned at 20 °C/0% RH (○) and 20 °C/45% RH (●), respectively.

The yield pressure, which is regarded as a measure of the densification of powder was calculated from the Heckel-plots (Fig. 2) using the linear range between 11 and 110 MPa ( $r^2=0.992$ ) for the tablets from 0% RH, and 7 to 120 MPa ( $r^2=0.996$ ) for the tablets made from 45% RH. The yield pressures of inulin conditioned at 0% RH and 45% RH were found to be 68 MPa and 39 MPa, respectively. The decreased yield pressure with increased humidity can be ascribed to the plasticizing effect of water, which facilitates deformation. Similar results have also been found for amorphous lactose [21] and amyloextrins [22]. In Fig. 3 the tensile strength as a function of compaction pressure is shown. The tablets made from material stored at 45% RH show a good linear behaviour up to 130 MPa, while tablets made from material stored at 0% RH demonstrate a more scattered behaviour, which can be explained by the capping behaviour mentioned above. At compaction pressures above 90 MPa the tensile strength of the tablets made from the material equilibrated at 45% RH decreased.



**Fig. 2** The  $-\ln$  (porosity under pressure) versus compaction pressure for freeze-dried inulin conditioned at 20 °C/0% RH (○) and 20 °C/45% RH (●), respectively.

Most likely, at these high pressures material densification with concomitant elastic energy storage occurred, resulting in internal non-visible capping. The reason for the increased tensile strength of tablets prepared from the moist material can again be found in the ability of absorbed water to act as a plasticizer. As a result, the degree of plastic deformation increases during compaction, and the lower porosity leads to a closer packing of the particles. This means that the available bonding surface within the tablet increases [23].



**Fig. 3** The tensile strength as function of compaction pressure for freeze-dried inulin conditioned at 20 °C/0% RH (○) and 20 °C/45% RH (●), respectively.

Because inulin conditioned at 20 °C/0% RH had poor compaction behaviour, tablets for the stability tests were compacted of inulin conditioned at 20 °C/45% RH. After 100 turns in the friabilator all tablets made from freeze-dried inulin compacted at 110 MPa were still intact. The friability of the tablets conditioned at 20 °C/45% RH was found to be 0.6%, which is within the requirements of the European Pharmacopoeia [19].

#### *Stability of alkaline phosphatase*

In a previous study [12] it was found that trehalose provided no protection of BIAP when stored at 60 °C/0% RH for six days, but when inulins were used the remaining activity was about 55%. However, in that study the ratio between BIAP/trehalose was 1/9 w/w, while in the present study the ratio was 1/19 w/w. The stability of BIAP incorporated in sugar glasses of either inulin or trehalose was tested by exposing powders and tablets to 60 °C/0% RH. For comparison, uncompact powders and tablets were also stored at 20 °C/0% RH. Tablets containing inulin were made from material conditioned at 20 °C/45% RH. The tablets containing trehalose were made from material conditioned at 20 °C/0% RH, because amorphous trehalose easily passes the glass transition temperature upon

exposure to humidified air [7]. Crystallization will be detrimental to the incorporated BIAP, since the protection will be completely lost.

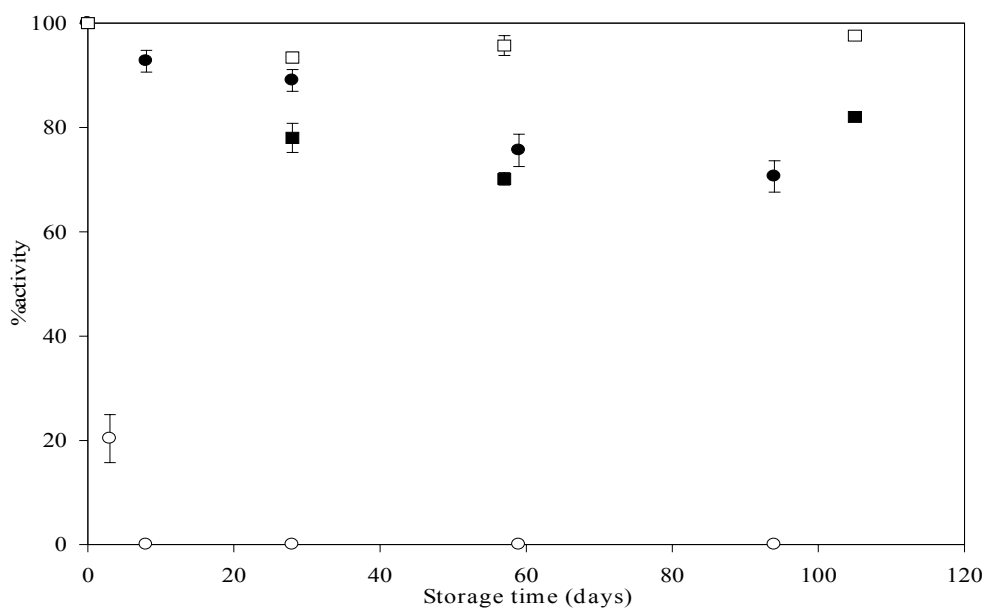
The enzymatic activity of BIAP after freeze-drying and the subsequent process steps was fully maintained in all cases where trehalose or inulin glasses were used (Table I) as protectant. When no protectant was used the activity of BIAP was almost completely lost after freeze-drying, indicating that both trehalose and inulin are excellent stabilizers during drying.

**Table I.** Remaining relative activity of BIAP after various process steps. Given is the enzymatic activity relative to theoretical values.

<i>Sugar</i>	Freeze-drying (%)	Grinding (%)	Pre-conditioning (%)	Compaction (%)
<i>Inulin</i>	108.9±0.4	110.3±0.3	103.8±1.1	99.5±2.8
Trehalose	107.5±3.5	101.1±2.3	102.8±2.3	99.9±0.8
No Protectant	5.4±2.1	n.a.	n.a.	n.a.

The appearance of the tablets made from inulin was different from the trehalose tablets. The surface of the trehalose tablets was not as smooth and they were very sticky. To minimize the moisture uptake the compaction was performed as rapidly as possible (less than a minute between weighing the material and compaction). However, during this procedure the freeze-dried amorphous trehalose material probably absorbed some water, which might cause a suppression of the T<sub>g</sub> [24, 25].

In Fig. 4 the results of the stability test are given. Already after 3 days at 60 °C the activity in tablets prepared from trehalose had dropped to 20.3±4.6% of the original value. Moreover, after 8 days storage at 60 °C the tablets made from trehalose had turned a little yellow and they also had a foul smell. On the other hand, the tablets were less sticky than immediately after compaction. In addition, the enzymatic activity of the BIAP completely disappeared. This is in sharp contrast to the trehalose powder, which shows a good stability. On the other hand, the BIAP in the tablets made from inulin showed about the same stability as the powder (75±3% activity after 3 months at 60 °C/0% RH).



**Fig. 4** The remaining enzymatic activity of sugar-incorporated BIAP (compacted and non-compacted) after storage at 60 °C. Inulin tablet (●), Trehalose tablet (○), Inulin powder (■), Trehalose powder (□).

When the stability of both powders was compared the BIAP was somewhat more stable when it was incorporated in trehalose. The enzymatic activity of BIAP for the samples stored at 20 °C/0% RH showed no loss of activity during the test period (Table II), indicating that both trehalose and inulin are excellent stabilizers when stored at mild conditions such as 20 °C/0% RH.

**Table II.** Remaining activity of BIAP after storage of powders at 20 °C/0% RH.

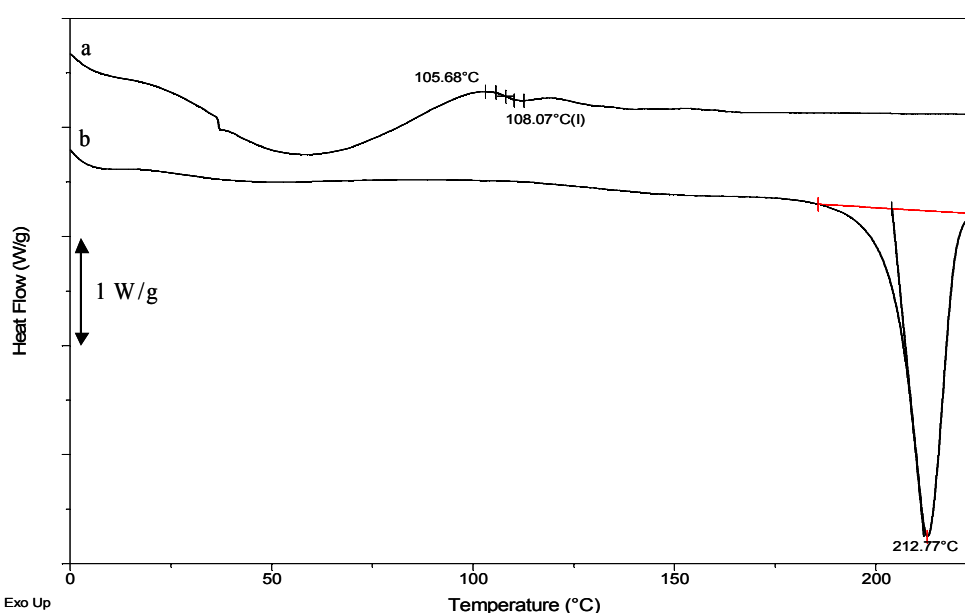
Days	Inulin/BIAP (%)	Trehalose/BIAP (%)
0	99.8±1.9	107.5±1.9
28	97.2±2.1	100.6±0.5
57	99.8±1.2	101.1±1.4
105	95.3±1.1	105.4±3.1

#### *Physical stability of trehalose and inulin glasses*

BIAP incorporated in trehalose completely loses its activity within eight days after compaction and storage at 60 °C. The fast disintegration of tablets containing BIAP incorporated in trehalose may be explained by the crystallization behaviour of trehalose. Factors such as moisture and

compaction may induce crystallization of amorphous sugars. As mentioned above, amorphous BIAP/trehalose became very sticky during the compaction process. Therefore, it is likely that the glass had partially turned into a rubber due to the compaction process and/or moisture uptake, i.e. the  $T_g$  had dropped to close to room temperature. As a result, the low  $T_g$  in combination with the compaction process and subsequent storage at 60 °C, crystallization of the lyophilized trehalose and consequently loss of protection of BIAP occurred.

This hypothesis is endorsed by the result of the DSC analysis showing an endothermic peak at 213 °C (Fig. 5), indicating that the material had fully turned into crystalline anhydrous trehalose [26]. The uncompacted trehalose, on the other hand, did not show any melting at 213 °C, while a  $T_g$  at 108 °C was observed indicating the existence of amorphous material (Fig. 5).



**Fig. 5** DSC of freeze-dried trehalose/B IAP immediately after compaction (a) and after compaction and storage at 60 °C/0% RH (b), respectively.

Pure amorphous trehalose has previously been reported to have a  $T_g$  of 115 °C [27] and 119 °C [26], respectively. The somewhat lower  $T_g$  found here can be ascribed to the presence of BIAP and/or buffer components in the sample.

Crystallization caused by compaction was not observed for the freeze-dried inulin that had been stored at 45% RH before compaction. Obviously, inulin does not seem to crystallize as easily as trehalose, since the T<sub>g</sub> was still clearly detectable at 151 °C (Fig. 6).

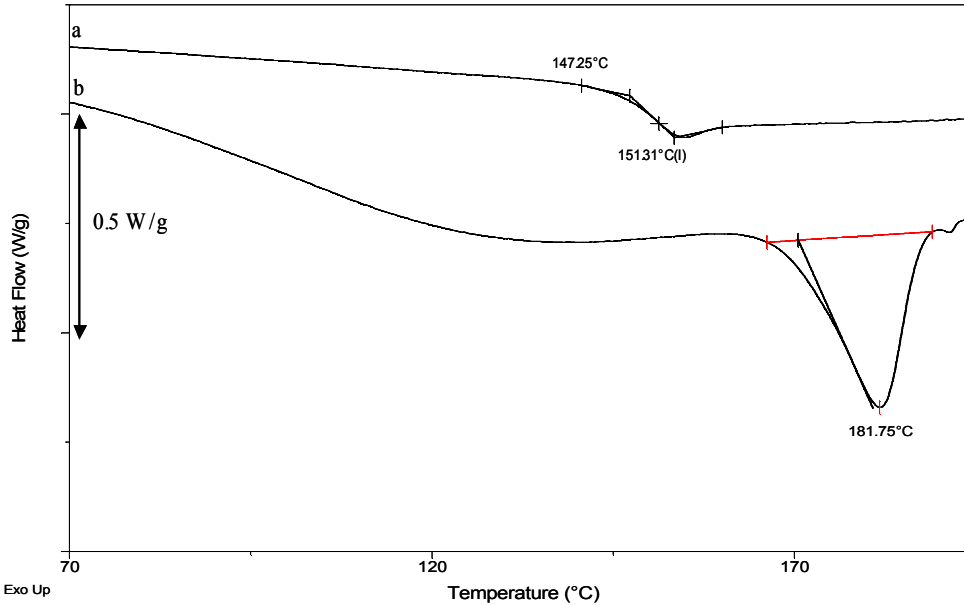


Fig. 6 DSC of amorphous inulin (a) and crystalline inulin (b).

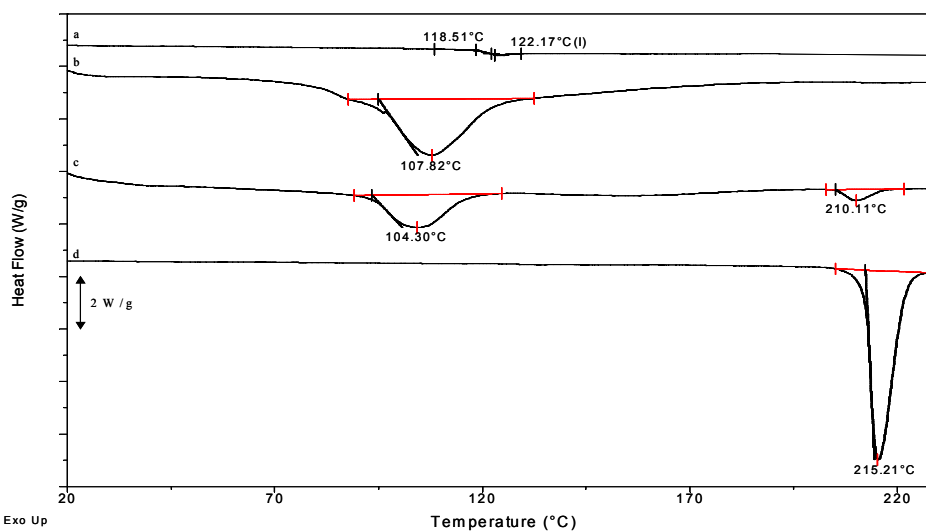
Crystalline inulin was also measured by DSC and the result is shown in Fig. 6. As can be seen no T<sub>g</sub> is measured but only a melting peak at 182 °C. In a previous study it has also been shown that amorphous inulin can absorb much higher amounts of water, compared to amorphous trehalose, without showing crystallization at room temperature [12].

This was further investigated by freeze-drying trehalose and inulin and compaction of powders, followed by storing the powders and compacts under various conditions. In Table III the results of the investigation of trehalose are given. As can be seen, freeze-dried trehalose remains amorphous when stored under dry conditions.

**Table III.** Compilation of DSC data of trehalose. <sup>a</sup>When exposed to humidified air, the  $T_g$  will decrease due to the uptake of water. However, DSC was performed in open pans. As a result, absorbed water will evaporate during the measurement and a  $T_g$  of the dry material is measured.

Preconditioning		DSC		Conditioning after compaction		DSC	
RH (%)	T (°C)	$T_g$ (°C)	$T_m$ (°C)	RH (%)	T (°C)	$T_g$ (°C)	$T_m$ (°C)
0	20	123	-	0	20	122	-
0	60	122	-	0	60	123	-
33	20	122 <sup>a</sup>	-	33	20	-	104/210
33	60	-	215	n.a.	n.a.	-	-
45	20	-	108	n.a.	n.a.	-	-

For these samples only a  $T_g$  at ca 122 °C was found (see also Fig 7). This  $T_g$  is in good agreement with what others have found for amorphous trehalose [26]. When the freeze-dried trehalose was stored at 20 °C/33% RH it still remained amorphous, but when this material was compacted it crystallized as both trehalose dihydrate and trehalose anhydrate, which could be concluded from melting peaks at 104 °C and 210 °C, respectively (see also Fig. 7). If the freeze-dried trehalose was conditioned at 20 °C/33% RH and then compacted and stored at 60 °C/33% RH it crystallized as trehalose anhydrate. For this material no  $T_g$  was seen, but only a melting signal at 215 °C was detected in the DSC measurement (see also Fig. 7). After storage at 20 °C/45% RH the freeze-dried trehalose powder turned into a hard cake, and when subjected to DSC melting of trehalose dihydrate was detected at 108 °C (see also Fig. 7).



**Fig. 7** DSC of freeze-dried trehalose stored at 20 °C/0% RH (a), freeze-dried trehalose stored at 20 °C/45% RH (b), freeze-dried trehalose stored at 20 °C/33% RH followed by compaction and storage at 20 °C/33% RH (c), compacted freeze-dried trehalose stored at 60 °C/33% RH (d), respectively.



When all these results are taken together it is evident that moisture and elevated temperature facilitates crystallization of amorphous trehalose. Moreover, crystallization is also induced when the powders are compacted. In previous studies of the crystallization behaviour of trehalose it was found that amorphous trehalose that was humidified at RHs below 44% would not crystallize due to lack of water [7, 8]. However, if the amorphous trehalose was exposed to RHs above 44% it rapidly crystallized. The crystallization of trehalose has previously been claimed to be the reason for the loss of protection of lactase [8]. The results that were found in our investigation of the crystallization behaviour of amorphous trehalose in combination with the findings of others support our assumption that crystallization of trehalose indeed was the reason for the loss of protection and subsequent degradation of the BIAP during the stability study.

For inulin it was found that it remained amorphous in climates up to 45% RH, and no collapse of the material was visible. Even when inulin was stored at 60 °C/33% RH it did not crystallize, not even after compaction. These results clearly show that inulin has a lower tendency to crystallize than trehalose, which further explains why the alkaline phosphatase was more stable when freeze-dried with inulin than with trehalose. It is evident that in order to achieve a good stabilization of a protein the use of inulin as stabilizer is to prefer above trehalose.

#### **6.4 Conclusions**

Tablets with adequate tensile strengths and low friability can be made of amorphous inulin. The moisture content in the material affected the compaction properties of inulin. In addition, the results indicate that tablets can be made of proteins incorporated in the inulin glass without loss of activity during compaction and subsequent storage, which is not the case for trehalose.

Our assumption that crystallization of trehalose was the reason for the lost activity of BIAP was also confirmed. Indeed, it was found that amorphous trehalose started crystallizing when exposed to various process conditions, such as increased RH, increased temperature and compaction, a phenomenon that was not found for amorphous inulin. These findings point to the superiority as stabilizer of amorphous inulin over amorphous trehalose. Inulin can be processed under less tight conditions, i.e.

amorphous inulin can be exposed to higher RHs than amorphous trehalose, which readily crystallizes. Inulin is clearly a better choice than trehalose when solid dosage forms are prepared.

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