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Title

Comparison of melibiose and trehalose as stabilising excipients for spray-dried β-galactosidase formulations

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Abstract

Spray-dried protein formulations commonly require stabilising excipients to prevent protein degradation during processing and storage, and trehalose has been commonly used. The purpose of this work was to evaluate melibiose in spray-dried protein formulations in comparison to trehalose. The protein-activity-preserving efficacy, process behaviour and storage stability were studied. Spray drying of β-galactosidase was carried out using different process temperature, drying air flow and feed liquid atomisation settings. Both melibiose and trehalose reduced protein activity loss during drying. A decrease in activities was observed when the process temperature exceeded a threshold temperature. During storage (30 days at 18% RH and 20 or 40 °C), the formulations dried below this threshold temperature showed no further activity loss, and the stabilising efficacy of the two disaccharides was equal. With higher process temperatures, the remaining protein activities after storage trended higher with melibiose formulations. All formulations remained amorphous. The powder yields of melibiose formulations were similar to trehalose. There was a difference in residual moisture contents, with melibiose formulations giving drier products. In conclusion, protein formulations with melibiose could be spray dried into amorphous powders that were physically stable, contained lower moisture contents and protected protein activity at least as well as trehalose formulations.

Keywords

spray drying, protein stability, excipients, melibiose, trehalose

1. Introduction

The nature of protein structure is typically complex and labile, making these biomolecules susceptible to various chemical and physical instabilities (Manning et al., 2010). In case of therapeutic proteins, degradation of the native structure can cause unacceptable changes in pharmaceutical properties such as loss of bioactivity or increased potential for triggering adverse immunological responses (Jiskoot et al., 2012; Schellekens, 2002). One strategy for overcoming

the limited stability of proteins and to achieve acceptable shelf life as pharmaceutical products, is the preparation of solid protein formulations by drying (Abdul-Fattah and Truong, 2010; Wang, 2000). However, drying processes and storage in the dried state can also be harmful to proteins and stabilising excipients are usually needed to protect against the drying stresses (Chang and Pikal, 2009; Ohtake et al., 2011).

Sugars which are able to both hydrogen bond with the protein as well as form a rigid, amorphous sugar matrix structure, are often efficient stabilisers (Arakawa et al., 2001; Mensink et al., 2017). Disaccharides have been found suitable in many cases, and trehalose and sucrose are commonly used as stabilising excipients in commercial dried protein formulations (Mensink et al., 2017; Wang et al., 2007). For storage stability, it is essential that the stabilising excipient does not crystallise, but remains amorphous and in a single phase with the protein molecules (Izutsu et al., 1993; Mensink et al., 2016). There have been issues with crystallisation of trehalose and sucrose, which can result in protein unfolding and aggregation (Eriksson et al., 2002; Ohtake and Wang, 2011; Tzannis and Prestrelski, 1999; Vandenheuvel et al., 2014). It is valuable to investigate new options because the list of protein-stabilising excipients currently available is limited and they do not always provide sufficient stability.

Protein formulations can be dried using different drying technologies, of which lyophilisation has been the most common choice (Abdul-Fattah et al., 2007; Ohtake et al., 2011; Wang, 2000), but today spray drying is an increasingly used method in the biopharmaceutical industry (Abdul-Fattah and Truong, 2010; Walters et al., 2014). Spray drying offers advantages including fast and energy-efficient processes, as well as control over particle properties. In the process, a liquid feed is transformed into a powder through atomising the liquid into small droplets and exposing them to heated air, where the droplets dry rapidly. Powder properties can be controlled by several process parameters including process temperature, liquid feed rate, atomisation and drying air flow settings, as well as feed solution variables (Cal and Sollohub, 2010; Paudel et al., 2013; Singh and Van den Mooter, 2016). Trehalose has been a standard excipient for spray-dried proteins because

of its stabilising efficacy and good processability compared to e.g. sucrose (Adler and Lee, 1999; Maury et al., 2005a).

The aim of this work was to evaluate the potential of melibiose as a stabilising excipient in spray-dried protein formulations. Melibiose is a disaccharide, naturally present in e.g. honey and many plants, but industrially produced by enzymatic hydrolysis of raffinose, a trisaccharide found in agricultural by-products, such as cottonseed and bean pulp (Kanters et al., 1976; Zhou et al., 2017)**.** The molecular structures of melibiose and trehalose are presented in **Fig 1** and some properties are compared in **Table 1**. Trehalose has the highest glass transition temperature (T_g, 120 °C) among disaccharides, which mostly range between 65-100 °C (Cesàro et al., 2008). Melibiose also has a high T_g for a sugar, and it has advantages in spray drying compared to other carbohydrates, including sucrose and isomalt (Lipiäinen et al., 2016). Melibiose has shown potential in lyophilised protein formulations, and even though it is a reducing sugar, no evidence of Maillard reaction-based protein degradation was observed during a three-month study with monoclonal antibodies (Heljo et al., 2013; Heljo et al., 2011).

Spray drying of protein formulations with melibiose as stabilising excipient has not been reported before. Therefore, the objective of this study was to investigate the protein-stabilising efficacy provided by melibiose during spray drying and storage in the dried state. Another objective was to evaluate the process behaviour of protein formulations containing melibiose. The effect of process parameters on powder recovery and properties, ability to preserve protein activity, and storage stability of protein formulations containing melibiose were compared to formulations containing the standard excipient, trehalose.

2. Materials and methods

2.1 Materials

The enzyme β-galactosidase, also called lactase, which is utilised as a dietary supplement for lactose intolerance, was used as a model protein. The protein (Lactase DS, from *Aspergillus oryzae*, EC number 3.2.1.23) was kindly provided as a gift by Amano Enzyme Inc. (Nagoya,

Japan). The evaluated disaccharide excipients, melibiose (M5500, Sigma-Aldrich, Slovakia) and trehalose (T9531, Sigma-Aldrich, USA), were purchased from Sigma-Aldrich. Reference experiments were performed using maltodextrin (Maltrin M180, dextrose equivalent of 18, Grain Processing Company, USA) and erythritol (Sigma-Aldrich, USA). Maltodextrin is a polymer, and it was used as a representative of a higher molecular weight (MW approx. 1000) and T_g (about 150 °C) compound compared to the disaccharides. Erythritol is a monosaccharide-based sugar alcohol with a closer molecular weight (112.1 g/mol) to the disaccharides, but a lower T_g (-42 °C) and very high propensity to crystallise.

2.2 Sample preparation

The received protein powder was rehydrated, filtered through a 0.2 µm filter (Acrodisc, Pall Corp, USA), and the pre-existing small-molecular-weight formulation components were purified using desalting columns (PD-10, GE Healthcare, USA). The purified product was mixed with 10% excipient-water solution (ad 100 ml). The protein concentration was measured using UV spectrophotometry (A280 nm, UV-1600PC, VWR, China), and the preparation was spray-dried during the same day. The final protein concentrations in the solutions were 0.39 ± 0.04 mg/ml, giving approximately 1:250 protein:excipient weight ratio.

2.3 Spray drying

The protein-excipient solutions (100 ml) were spray dried using a Büchi B-191 Mini spray drier (Büchi Labortechnik AG, Switzerland), with a two-fluid nozzle and co-current operation mode. The instrument was equipped with cooling water circulation for the nozzle and nitrogen supply as the atomising gas.

A 2^3 full factorial design was planned using the modelling and experimental design software MODDE (Umetrics AB, Sweden), with 140 and 180 °C as the low and high levels for inlet temperature, 500-800 L/h for the atomising gas flow rate, and 80-100% for the aspirator rate. The aspirator controls the drying air flow rate, and the studied operation range resulted in 25.5-31.0 m^3/h volumetric flow rate as measured by a Testo 425 air flow meter, Humitec, Finland. The liquid

feed rate was kept constant at 4.8 mL/min in order to standardise the process duration and heat exposure of the product (approx. 20 minutes). The experiments with different process parameters were carried out in randomised order.

The dry particles were separated from the drying air stream by a cyclone (Büchi standard cyclone). Powders that were recovered from the product collection vessel and the bottom of its metallic lid were considered as the yield (mass percentage compared to the initial mass of solids), and transferred into glass vials for analysis and storage. The powder handling and sample preparation for analysis was carried out at 22±1 °C and 23±3 % RH.

2.4 Storage stability studies

The powders were stored in glass vials, inside desiccators at two different temperature conditions: room temperature (20 °C) and elevated temperature (40 °C), both at 18 % RH. The samples were analysed during and at the end of a 30-day storage study.

2.5 Protein activity assay

Protein stability was evaluated based on remaining activity. The activities were determined by an enzymatic assay for β-galactosidase (according to Sigma quality control test procedure 11/01, based on (Bahl and Agrawal, 1969; Borooah et al., 1961)), which is a spectrophotometric o-nitrophenyl-β-D-galactopyranoside (ONPG; substrate for β-galactosidase) cleavage rate test. The activity was measured from each sample after protein purification and mixing with the excipient solution: this was considered the initial activity and assigned as 100% relative activity for the corresponding experiment. To determine the remaining activity after spray drying and storage, the samples were rehydrated and diluted to the initial concentration. The relative activity of the processed or stored sample compared to the initial activity was considered the remaining protein activity. The assay was performed with duplicate samples, and the measurements before and after spray drying were performed in parallel.

2.6 Characterisation of powder properties

Thermal analysis of the powders was performed by differential scanning calorimetry (DSC 823e, Mettler-Toledo GmbH, Switzerland). Duplicate samples (approx. 5 mg) were sealed in aluminium pans, equilibrated at 25 °C for 3 min and heated to 200 °C at a 10 °C/min rate, with nitrogen as purge gas (50 ml/min). The glass transition temperatures (T_g) were determined as the midpoints of the transitions using STARe software (Mettler-Toledo GmbH, Switzerland).

Sample crystallinity was investigated using X-ray powder diffractometry (XRPD, Bruker D8 Advance, Bruker Axs Inc, USA), with CuKα radiation (λ=1.54 Å). The samples were flattened into aluminium holders, and scanned over the 5-40° angular range (2θ) at a rate of 0.1°/s.

The moisture contents of the powders were determined using the DSC results and water activity measurements (a_w). The a_w measurements were carried out using an AquaLab 3 water activity meter (Decagon Devices, USA), where the water activity was determined at 25 °C and in triplicate for each sample. The residual moisture content was calculated based on the measured a_w and T_g values, by using the correlation between each of these values and the sample water content. This was based on linear fits observed with data from previous studies: 1) between the measured Karl Fischer titration and a_w values (R^2=0.88 for melibiose and R^2=0.67 for trehalose), and 2) between the measured KF and T_g values (R^2=0.67 for melibiose and R^2=0.53 for trehalose). These two regression models were used to predict the powder moisture contents, separately based on the measured a_w and T_g values. The average of these two determinations was recorded as the moisture content. The method was verified with Karl Fischer titration (V30, Mettler-Toledo AG, Switzerland), and the error was ±0.3%.

The particle morphology of the powders was imaged by scanning electron microscopy (SEM) using a FEI Quanta 250 FEG system (FEI Inc, OR, USA). The samples were fixed onto carbon tapes and sputtered with platinum (Quorum Q150TS, Quorum Technologies, UK).

2.7 Data analysis

The results were evaluated with MODDE (Umetrics AB, Sweden). Partial least squares (PLS) fitting was used to identify relationships (covariance) between the process factors and the measured responses. The models were fitted using only the significant terms (coefficients), judged by their uncertainty levels (excluding the ones ranging across y=0)**,** in order to maximise the predictability (by maximising Q^2) and reduce the risk of overfitting (by minimising the difference between the model fit R^2 and Q^2). The statistical significance was confirmed by analysis of variance (ANOVA), defined at $p<0.05$.

3. Results and discussion

3.1 Protein activity preservation during spray drying

Both melibiose and trehalose provided protection for protein activity during the spray drying processes (**Table 2**). The remaining protein activities in the powders containing melibiose or trehalose were between 65-90%. In contrast, formulations with maltodextrin or erythritol showed clearly reduced activities: 40% remaining activity was observed with maltodextrin, and only 3% activity was remaining with erythritol after spray drying (inlet temperature 160 °C, outlet temperature 83-84 °C).

The remaining protein activities decreased when higher inlet temperature or aspirator rate was used, as indicated by the PLS model (MODDE, at confidence level > 0.95). Both the inlet temperature and the aspirator rate (drying air flow rate) contributed to the outlet temperature, with the inlet temperature having a stronger impact. The outlet temperature can be considered as the maximum temperature to which the end product is exposed to (Cal and Sollohub, 2010).

The atomising gas flow rate did not affect the remaining protein activities. Higher pressure atomisation results in stronger mechanical stresses which can be harmful to proteins, but no evidence of this was seen in the β-galactosidase activity levels. A similar result has been reported in an earlier study, where atomisation by ultrasound nebulisation had no effect on β-galactosidase activity (Genina et al., 2010).

Thus, the major factor in β-galactosidase activity loss during spray drying was the process temperature. A clear decrease in activities was seen after the outlet temperature reached a threshold level of approximately 90 °C (**Fig 2a**). The spray drying experiments with a lower level for inlet temperature (140 or 160 °C) resulted in outlet temperatures in the range of 70-86 °C, and the protein activities were stable in this group of experiments. In contrast, in the experiments with inlet temperature at the high level (180 °C) and outlet temperatures at 88-103 °C, the remaining protein activities decreased in a temperature-dependent manner. In both temperature groups, the remaining protein activities showed slight trends towards higher values with melibiose than with trehalose formulations (**Fig 2b**). However, the difference between the protective efficacies of the two excipients was not statistically significant.

Neither of the formulations showed full recovery of protein activity after spray drying. In a previous study by Bürki et al., β-galactosidase activity could be fully protected by trehalose during spray drying processes with outlet temperatures of 70-80 °C (Bürki et al., 2011). This inconsistency in results can be caused by the difference in process durations, which were shorter (approx. 5 min) in the work by Bürki et al. compared to the ones in this study (approx. 20 min). The powder was exposed to elevated temperature in the product collection vessel during the full process time, and therefore the process conditions were particularly harsh in the experiments carried out in the present study.

Nevertheless, the stabilising efficacy of both disaccharides evaluated in this work is evident when compared to the reference excipients. Even though these experiments with one polymer and one monosaccharide alcohol cannot be regarded as a representative study on the topic, the results here are consistent with earlier reports and discussion indicating the generally good stabilising ability of disaccharides compared to larger molecules, which remain amorphous but are structurally bulky, or compounds that crystallise (Arakawa et al., 2001; Izutsu et al., 1993; Mensink et al., 2017; Souillac et al., 2002; Tonnis et al., 2015). It can be expected that melibiose is able to provide full protein activity preservation during spray drying, by adjusting the process settings.

Overall, our results showed that best activity preservation with β-galactosidase was obtained when adjusting the inlet temperature and the aspirator rate to lower settings. Melibiose was at least as good as trehalose in stabilising protein activity during spray drying.

3.2 Powder yield

All studied process settings were suitable for producing spray-dried protein powders containing either melibiose or trehalose (**Table 3**). Powder yields were in the range of 39-74%, and similar for the two formulations. For melibiose, the powder recoveries were clearly higher compared to drying the sugar without protein, where lower yields (18-29%) have been observed when drying at similar temperatures (Lipiäinen et al., 2016). The small protein addition (1:250 w/w) resulted in increased melibiose yields, but such an effect was not observed for trehalose. Considering the feasibility of spray drying protein formulations containing melibiose, it is a promising result that the process parameters could be selected more freely compared to spray drying pure melibiose.

The powder yields depended on the atomising gas flow rate and inlet temperature, with the former having a stronger influence. An increase in atomising gas flow rate resulted in higher yields (**Fig. 3a**) and higher process temperatures reduced the yields (**Fig 3b**). Both parameters affect the drying and temperature of the product. Reduced powder yields can be observed when drying is not sufficient before impact with the dying chamber wall, or when the temperature of amorphous powders reaches the so-called sticky point temperature, also resulting in particle adhesion to the drier walls (Maury et al., 2005b). The sticky point is a complex and controversial topic, but it may be dependent on the T_g and the moisture distribution in the drying droplets/particles, and sticky behaviour has been observed when process temperature exceeds the material T_g by approx. 10-20 °C (Adhikari et al., 2009; Bowen et al., 2013; Maury et al., 2005b). With melibiose, the process temperature had a more pronounced effect on yield than with trehalose. This can relate to the lower T_g of anhydrous melibiose compared to trehalose. For trehalose, the yields were more dependent on the atomising gas flow rate and higher powder recoveries correlated with drier powders.

The aspirator rate did not affect the yields, and equal amounts of powder were collected over the studied aspirator setting range. The separation efficiency in the cyclone is dependent on the air flow rate and too low rates can reduce the yields (Cal and Sollohub, 2010). In this work, the aspirator rate could be reduced from 100% (31 m^3/h) to 80% (26 m^3/h) without a negative impact on the powder yield.

The differences in the powder yields between melibiose and trehalose were small and the observed yields were good for a mini-scale spray drier. Low powder collection yields have been a problem with spray drying, as they can often be below 50% when using benchtop model spray driers (Bowen et al., 2013). It is possible to further improve the powder recoveries by technical improvements in drier design and particularly with larger scale spray driers (Bowen et al., 2013).

3.3 Powder properties

The spray-dried protein formulations containing melibiose or trehalose produced smooth, spherical particles (**Fig 4**). The small protein addition (1:250 weight ratio) did not have an apparent impact on particle morphology, and the appearance resembled pure spray-dried disaccharide particles. When dried at the same process settings, the melibiose and trehalose powders were similar to each other.

All of the produced powders were amorphous and dry (**Table 3)**. The powders containing melibiose were drier (residual moisture content 1-2%) than the ones with trehalose (approx. 3%). The T_g range for melibiose powders (69-90 °C) was similar to the trehalose powders (68-85 °C), regardless of the T_g difference between the anhydrous materials. This was because of the difference in water contents, with the plasticising effect of water reducing the T_g values.

There was a stronger dependence between the moisture contents of trehalose powders and the process parameters than there was with melibiose. The most significant factor was the atomising gas flow rate, and the residual moisture contents of trehalose powders were lower when higher atomisation rates were used. This dependence was not observed when melibiose was used. Melibiose formulations were consistently dry with all process parameters, showing only a minor

trend towards drier powders when higher aspirator rates and inlet temperatures were used. The melibiose powders were generally 2-fold drier, and at best 3-fold drier than trehalose powders, when using the same spray drying process parameters (**Fig 5**).

The results showed that the use of dry nitrogen as atomising gas allowed the production of very dry (1-2%) amorphous melibiose-protein powders by spray drying. Unlike with the trehalose formulations, where the residual moisture contents depended on the atomising gas flow rate (500-800 L/h), consistently dry powders were produced with melibiose at all studied process settings. This suggests that more efficient spray drying processes are possible for protein formulations with melibiose compared to trehalose.

3.4 Storage stability

The protein activities remained stable during the 30-day storage study, at both room temperature (+20 °C) and elevated temperature (+40 °C) (**Fig. 6**). The most significant contributor to the preservation of protein activity during storage was the spray drying process temperature. Most of the protein degradation had occurred during processing and further changes during the duration of the storage study were small.

The recorded remaining protein activity values trended higher for melibiose formulations compared to trehalose formulations. The difference was most pronounced with the samples that had been spray dried and stored at higher temperatures. This may suggest better stabilisation potential of melibiose, since high temperatures are stressful to the protein and solid-state stability. However, the overall difference between the stabilising efficacies of the two excipients was not statistically significant. These results show that melibiose was at least as good a protein-stabiliser during storage as trehalose.

Regarding physical stability, the powders remained nearly unchanged during the 30-day study. All samples were amorphous, indicated by the XRPD diffractograms with typical amorphous halos, as well as the presence of glass transitions in the DSC curves (**Fig 7**). Small decreases in the T_g values had occurred (**Table 4**), compared to the values recorded after spray drying (69-91 °C for

melibiose and 69-86 °C for trehalose). These changes reflected the small increases in the moisture levels of the powders. The changes were more apparent with the samples stored at elevated temperature.

Changes in the powders stored at the elevated temperature conditions (40 °C, 18% RH) were apparent form the DSC curves, where enthalpy recovery events became clearly visible (**Fig 7c and f**). Evaluation of relaxation behaviour from conventional DSC measurements is not straightforward (Kawakami and Pikal, 2005). Nevertheless, the increased enthalpy recovery events imply higher molecular mobility in the samples, which could lead to crystallisation and protein degradation.

The degree of the observed physical changes did not correlate with the measured protein activities after storage. Furthermore, the changes induced by the storage conditions were not sufficient to cause full crystallisation of neither melibiose nor trehalose. The presence of local crystallites in the amorphous matrices, as has been suggested for trehalose (Cesàro et al., 2008), cannot be ruled out based on these results. It is clear, however, that both melibiose and trehalose formulations remained physically stable and without fully crystallising for 30 days at 40 °C.

From the DSC curves it can be seen that melibiose did not crystallise even during the heating scan, contrary to trehalose (**Fig 7**). Water plays a central role in melibiose crystal structure (Kanters et al., 1976), and no anhydrate forms have been reported. The moisture levels in the powders remained low, which was likely to hinder crystallisation to any hydrate form. In contrast, trehalose can form anhydrates, and when conditions are favourable for crystallisation, the lack of water does not prevent it. Amorphous melibiose has also previously been shown to have a slow crystallisation rate, along with low molecular mobility, compared to other disaccharides (Heljo et al., 2012).

All of the amorphous melibiose-protein formulations, produced using different spray-drying process parameters, remained physically stable during the storage study. The crystallisation behaviour observed with the DSC experiments suggests that melibiose may be able to resist crystallisation better than trehalose. This physical stability makes melibiose a promising material for amorphous formulations based on low molecular weight excipients, such as spray-dried protein products.

4. Conclusion

Melibiose prevented protein activity loss during spray drying and storage at least equally well as the commonly used excipient trehalose. After the 30-day storage, melibiose formulations showed trends towards higher remaining protein activities than trehalose formulations when the formulations had been spray dried and stored at higher temperatures (i.e. in more stressful conditions for protein and solid-state stability), which suggests good storage stabilisation potential for melibiose. Melibiose is worth further studies to investigate long term stability. Spray drying processes with acceptable powder yields were possible with both excipients. Melibiose presented an advantage of more efficient drying, and the formulations containing melibiose had consistently two-fold lower moisture contents than the trehalose formulations. The powders remained physically stable and did not crystallise during the 30-day storage study, and it can be expected that melibiose formulations may present better stability in the amorphous form than trehalose formulations. Overall, melibiose showed several promising properties for spray-dried protein

formulations, namely protein-stabilising efficacy, efficient spray drying processes and physical stability of the amorphous products.

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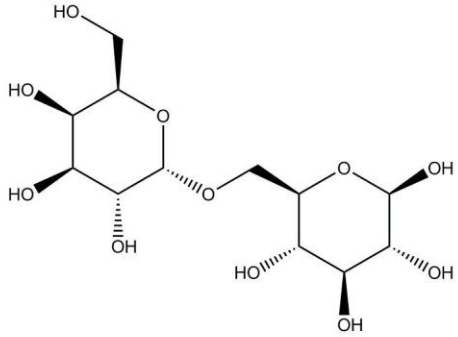

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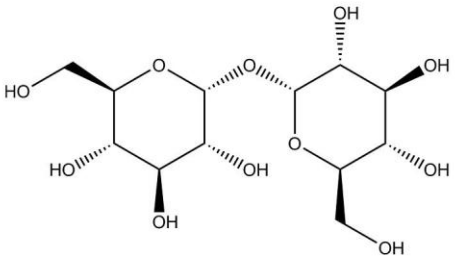

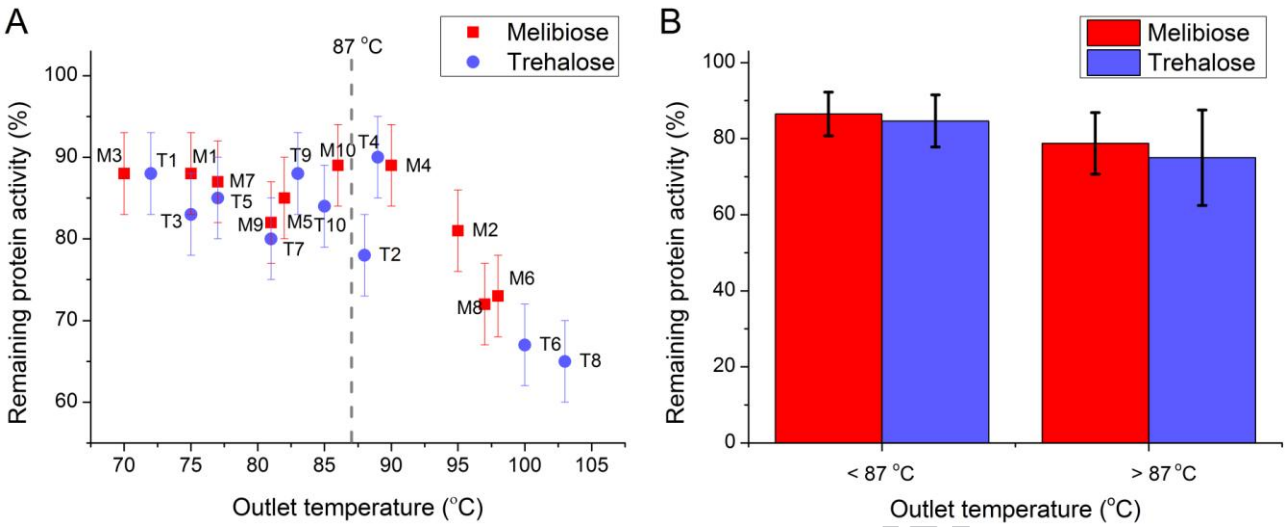

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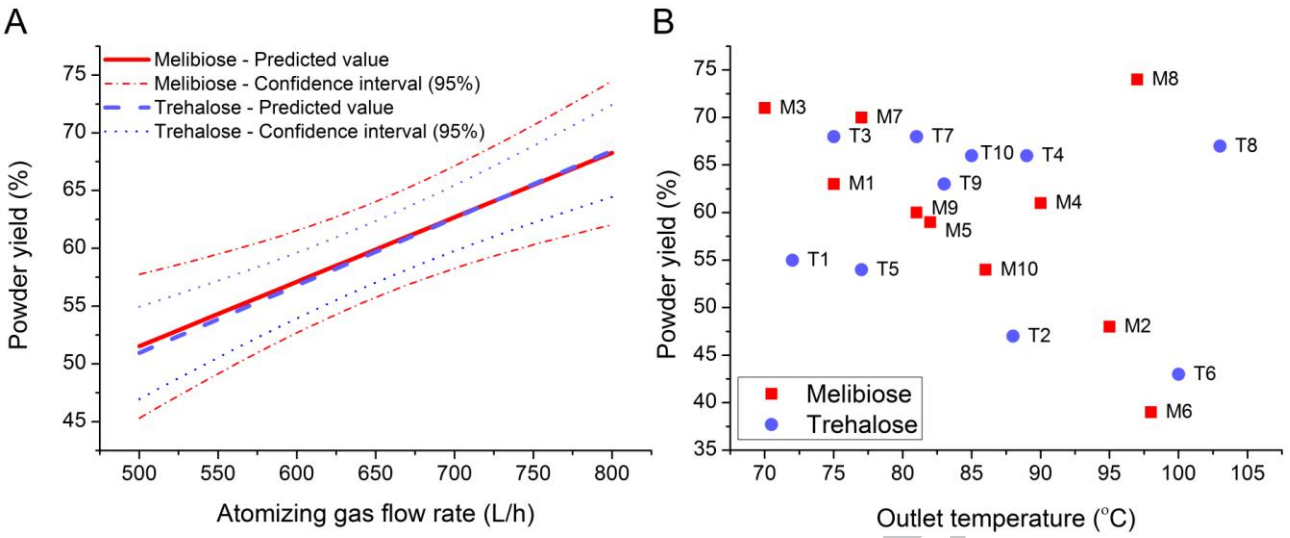

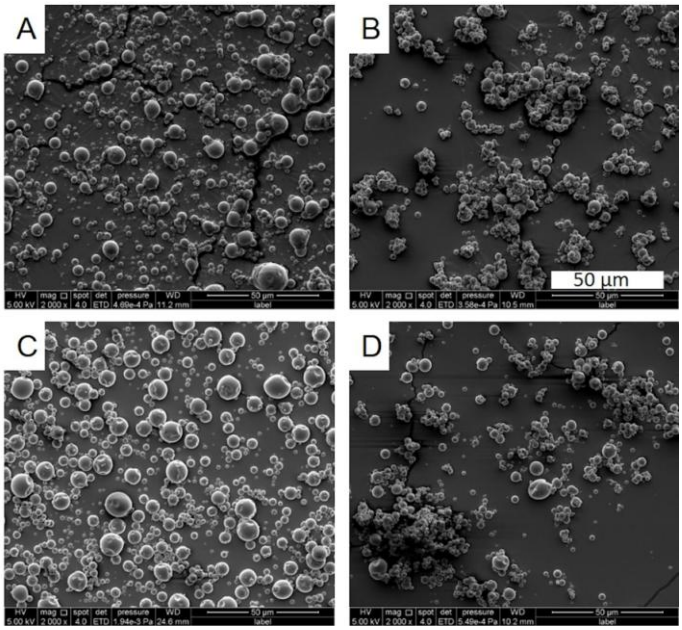

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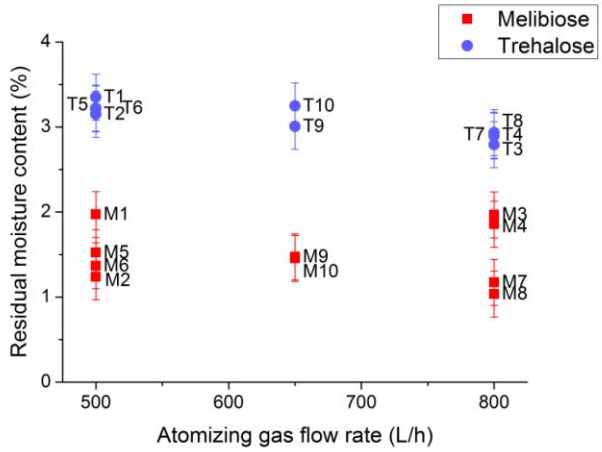

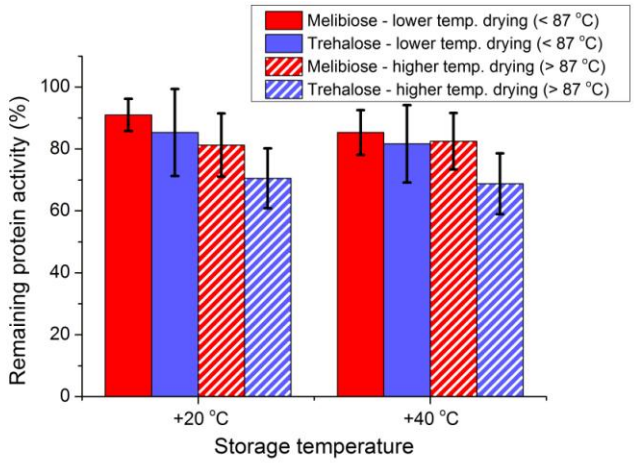

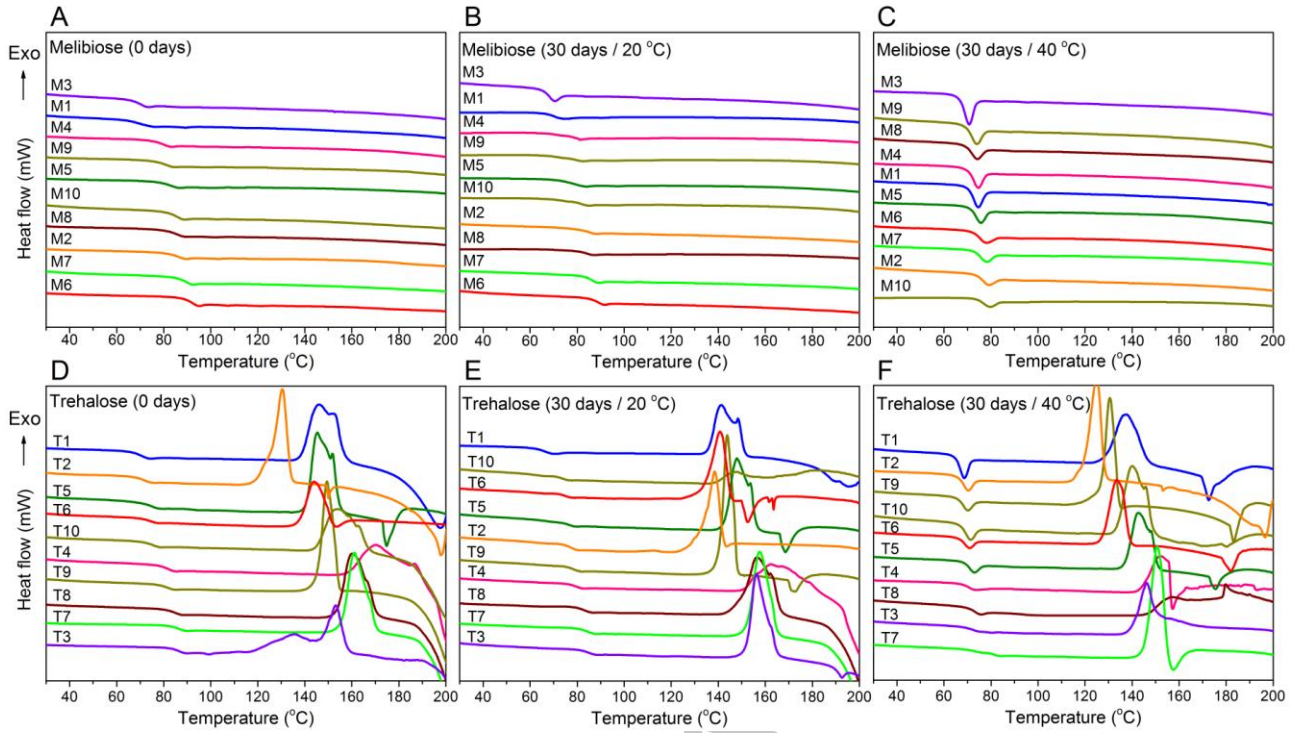

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Figure 1 Molecular structures of melibiose (A) and trehalose (B).

Figure 2. Remaining protein activities after spray drying as a function of outlet temperature (A) and the remaining protein activities, grouped based on process temperature (B). The lower temperature group (outlet temperature < 87 °C) had an inlet temperature of 140 or 160 °C and the higher temperature group (outlet temperature > 87 °C) had an inlet temperature of 180 °C. The error bars indicate the standard deviations. M=melibiose, T=trehalose and the number is the experiment number (see Table 2).

Figure 3. Effect of atomising gas flow rate (A) and outlet temperature (B) on powder yields. (A) presents predicted values when the inlet temperature is constant at 160 °C and the aspirator at 90% and (B) shows the observations, where M=melibiose, T=trehalose and the number is the experiment number (see Table 3).

Figure 4. SEM images of powders spray dried at 140 °C, 500 L/h, 80% (low settings) containing β-galactosidase with melibiose (A) or trehalose (C), and powders spray dried at 180 °C, 800 L/h, 100% (high settings) with melibiose (B) or trehalose (D). The scale bar (50 µm) is the same for all images.

Figure 5. Residual moisture contents of the spray-dried protein formulations with melibiose or trehalose as a function of atomising gas flow rate. The error bars indicate the standard deviations.

Figure 6. Remaining protein activities of the melibiose and trehalose formulations after 30 days of storage at +20 °C and +40 °C. The observations are divided according to the spray drying processes, into lower temperature processes (solid colour columns, inlet temperature 140-160 °C, outlet temperature <87 °C) and higher temperature processes (striped columns, inlet temperature 180 °C, outlet temperature >87 °C). The error bars indicate the standard deviations.

Figure 7. DSC curves of the spray dried protein powders containing either melibiose (A-C) or trehalose (D-F), measured immediately after drying (A,D), after 30-day storage at 20 °C (B,E) or after 30-day storage at 40 °C (C,F). The curves are ordered from top to bottom according to the Tg values. M=melibiose, T=trehalose, and the number is the experiment number (see Table 3).

Table 1. Comparison of melibiose and trehalose properties

	Melibiose	Trehalose	References
Description	Reducing disaccharide galactose and glucose with α-1,6- linkage	Non-reducing disaccharide two glucose units with α, α-1,1-linkage	(Kanters et al., 1976) (Cesàro et al., 2008)
Chemical formula	$C_{12}H_{22}O_{11}$	$C_{12}H_{22}O_{11}$	PubChem
Molecular weight (anhydrous)	342.3	342.3	
Solubility	soluble in water	soluble in water	(Lakio et al., 2013) (Ohtake and Wang, 2011)
T_g (anhydrous)	100	120	(Heljo et al., 2012) (Cesàro et al., 2008)
Crystalline forms and melting points (°C)	α-melibiose monohydrate (179-186), stable form β-melibiose dihydrate (85-86)	dihydrate (97-100; dehydration), stable form β form (205-215), stable anhydrous form α form (126) γ form[a] (120; transition to β form)	(Fletcher and Diehl, 1952) (Cesàro et al., 2008; Ohtake and Wang, 2011)

[a] Possibly a mixture of the dihydrate and β-anhydrate forms.

500 ***Table 2. Remaining protein activities after spray drying processes with melibiose or trehalose as excipient****. meas. 1 and meas. 2 refer to the results from the duplicate protein activity measurements.*

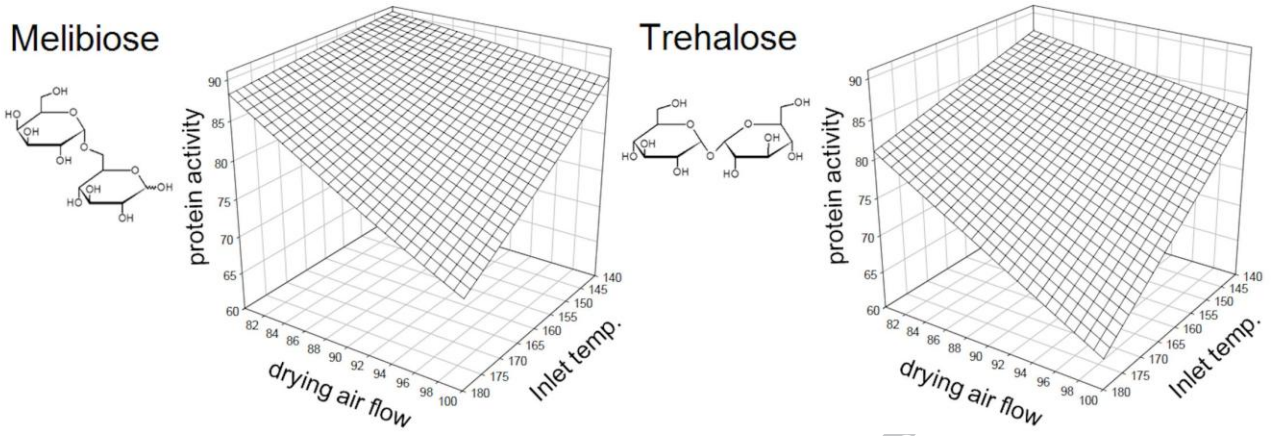
Process parameters				Responses					
				Melibiose			Trehalose		
Exp. no	Inlet temp.(°C)	Atomising rate (L/h)	Aspirator rate (%)	Outlet temp. (°C)	Protein activity (%) meas. 1	meas. 2	Outlet temp. (°C)	Protein activity (%) meas. 1	meas. 2
1	140	500	80	75	84	93	72	90	85
2	180	500	80	95	81	81	88	82	74
3	140	800	80	70	80	97	75	83	83
4	180	800	80	90	84	94	89	80	100
5	140	500	100	82	88	81	77	76	93
6	180	500	100	98	73	72	100	74	61
7	140	800	100	77	85	88	81	80	80
8	180	800	100	97	75	69	103	67	63
9	160	650	90	81	89	76	83	85	90
10	160	650	90	86	89	88	85	96	73

505 ***Table 3. Powder yields and properties of spray-dried protein formulations containing melibiose or trehalose****. The relationship between the aspirator rate and volumetric drying air flow rate was: 100% = 31.0 ± 0.8 m^3/h, 90% = 28.6 ± 0.4 m^3/h, 80% = 25.5 ± 0.5 m^3/h.*

Process parameters				Responses							
				Melibiose				Trehalose			
Exp no	Inlet temp. (°C)	Atomising rate (L/h)	Aspirator rate (%)	Outlet temp. (°C)	Yield (%)	Moisture content (%)	T_g (°C)	Outlet temp. (°C)	Yield (%)	Moisture content (%)	T_g (°C)
1	140	500	80	75	63	2.0 ± 0.4	69.8 ± 0.1	72	55	3.4 ± 0.3	68.4 ± 1.4
2	180	500	80	95	48	1.2 ± 0.0	85.3 ± 0.1	88	47	3.1 ± 0.1	79.3 ± 0.6
3	140	800	80	70	71	2.0 ± 0.5	69.4 ± 0.2	75	68	2.8 ± 0.0	84.9 ± 0.9
4	180	800	80	90	61	1.9 ± 0.0	77.2 ± 1.2	89	66	2.9 ± 0.1	81.1 ± 0.5
5	140	500	100	82	59	1.5 ± 0.1	80.3 ± 1.0	77	54	3.2 ± 0.1	75.2 ± 0.7
6	180	500	100	98	39	1.4 ± 0.1	84.7 ± 0.4	100	43	3.2 ± 0.1	74.9 ± 1.1
7	140	800	100	77	70	1.2 ± 0.2	88.2 ± 0.1	81	68	2.9 ± 0.1	84.4 ± 1.2
8	180	800	100	97	74	1.0 ± 0.2	89.8 ± 1.0	103	67	2.9 ± 0.1	85.3 ± 1.1
9	160	650	90	81	60	1.5 ± 0.2	79.3 ± 0.4	83	63	3.0 ± 0.0	80.1 ± 0.9
10	160	650	90	86	54	1.5 ± 0.2	84.3 ± 0.2	85	66	3.2 ± 0.0	74.9 ± 0.5

510 ***Table 4. Glass transition temperature, moisture content and water activity ranges of the spray-dried melibiose and trehalose protein formulations after storage for 30 days at 20 or 40 °C.***

	Melibiose		Trehalose	
	+ 20 °C / 18% RH	+ 40 °C / 18% RH	+ 20 °C / 18% RH	+ 40 °C / 18% RH
T_g (°C)	67.1-88.1	67.2-75.8	66.3-84.5	65.4-72.8
Moisture content (%)	1.1-2.1	2.0-2.5	2.9-3.6	3.5-3.9
water activity	0.027-0.067	0.085-0.139	0.042-0.100	0.108-0.168



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