تأثير حرارة التخزين في طاقة السطح الحرة لمساحيق الليزوزيم المجفف بالإرذاذ

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الملخص

خلفية البحث وهدفه: تُستخدم تقنيات التجفيف بالإرذاذ في تحضير مساحيق استنشاقية للإنْزيمات والبروتينات دون تَمَسُّخها. يُستفاد من معطيات طاقة السطح الحرة لهذه المساحيق بتقليل الزمن اللازم لتطوير مستحضراتها وتحسين جودة مَناشِق المساحيق. يمكن أن تتغير طاقة السطح الحرة لهذه المساحيق البروتينية الاستنشاقية، خلال التخزين، ومن ثَمَّ تؤثر في أدائها. و تؤثر عوامل عدة في مدى هذا التغيّر. ولكن درجة حرارة التخزين هي العامل الحَدِّيّ.

مواد البحث وطرائقه: طُورت طريقة تجفيف بالإرذاذ لتحضير مساحيق استنشاقية لليزوزيم مجففة بالإرذاذ (كنموذج للمساحيق البروتينية الاستنشاقية). استخدم الاستشراب الغازي العكوس لقياس طاقة السطح الحرة للمساحيق المحضرة قبل التخزين وبعده لكي توضح تأثير حرارة التخزين في طاقة السطح الحرة. ونظراً إلى البنية الفراغية البروتينية الضعيفة قد تتمسخ بدرجة حرارة التخزين، استخدمت تقنية المسح الحراري التفاضلي (DSC) و فحوص النشاط البيولوجيّ لتقييم سلامة البنية الفراغية لليزوزيم بعد التخزين في درجات حرارة مختلفة.

النتائج: تنقص زيادة درجة حرارة التخزين لكل من مكونات السطح اللاقطبية بنحو 12% والقطبية بنحو31% (ANOVA: P < 0.001) للمساحيق الاستنشاقية لليزوزيم المجففة بالإرذاذ. وهذا التناقص في مكونات السطح القطبية يؤدي إلى تناقص في قلوية السطح. يمكن أن تعزى هذه التغيّرات في طاقات السطح لتَكَدُّس جزيئات اليزوزيم المُتَمَسِّخة إلى جانب بعضها بعضاً من خلال المناطق الغنية بالإلكترونات الموجودة على سطح المساحيق.

الاستنتاج: يُنصح بتخزين المساحيق البروتينية في درجة حرارة منخفضة، على سبيل المثال 5 درجة مئوية، وذلك لمنع أي تغيّر في طاقة السطح الحرة التي تعدُّ عاملاً ذا تأثير حَدِّيّ في أداء هذه المساحيق.

كلمات مفتاحية: مسحوق بروتين استنشاقي، التجفيف بالإرذاذ، طاقة السطح الحرة، استشراب غازي عكوس، حرارة التخزين.

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The Effect of Storage Temperature on the Surface free Energy of Spray Dried Lysozyme Powders

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Abstract

Background & Objective: Spray drying techniques are used to produce inhaled powders of enzymes and proteins without denaturation. The surface free energy data of these powders is used to reduce the time of formulation development. During storage, the surface free energy of the inhaled protein powders could change and so their performance will be affected. Many factors affect the limit of this change. Storage temperature is the critical factor.

Material& methods: A spray drying method was optimized to prepare inhaled spray dried lysozyme powders. Inverse gas chromatography was utilized to measure the surface free energy of the prepared powders pre and post-storage to detect the effect of storage temperature on the surface free energy. Differential Scanning Calorimetry and biological activity tests were used to evaluate the integrity of lysozyme conformation after storage at different storage temperature.

Results: Increasing the storage temperature decreased both dispersive and specific surface components of the spray-dried lysozyme powders significantly (ANOVA: $P < 0.001$) up to ~12% and up to ~31 % after storage for one year at 40 °C, respectively. The overall decrease in the polar components resulted in a reduction of the surface basicity. These decreases in the surface energetics would be due to the aggregation of denatured lysozyme molecules at the powder surface via hydrophobic regions rich with electrons. The same results were found after storage for one year at room temperature, but the extents of changes were smaller.

Conclusion: It is advisable to store protein powders at 5°C to inhibit the changes in their surface free energy which is the crucial factor affecting their performance.

Key words: Inhaled protein powder, spray drying, surface free energy, inverse gas chromatography, storage temperature

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Introduction

Spray drying techniques have been successfully used to produce particles of heat-sensitive biological materials like enzymes and proteins without denaturation [(1)], as the cooling effect of solvent evaporation, which is usually water, protects the protein molecules from heat degradation [(2)]. Many protein powders for inhalation have been produced using the spray drying techniques. For example, respirable powders of recombinant human granulocyte-colony-stimulating factor (rhG-CSF) [(3)], insulin [(4)], and bovine serum albumin [(5)] have been successfully prepared. These spray dried particles have many advantages for delivering via the lungs. This is because the spray dried particles are spherical and hollow; therefore they have lower densities and consequently lower aerodynamic diameters. Spray dried particles with a spherical shape had a higher fine particle fraction deposition compared with angular jet milled ones having the same size [(6)].

The powder surface free energy data can be used to reduce the time of formulation development and enhance the quality of powder inhalers [(7)]. This is because the surface free energies of the materials incorporated inside inhalation devices; Pressurized metered dose inhalers (pMDIs) or Dry powder inhalers (DPIs) can crucially affect the inhalation formulations' performances, as they control the powder aggregation process, the ease of powder dispersibility, the adhesion and removal of protein powders to/from containers and carriers, and the dispersion of particles in liquefied propellants [(8-10)]. The mixing performance of a mixture of DPIs and the powder flowability can be also predicted form the surface free energy data [(11)]. Moreover, the generated static charges of particles by triboelectrification have been related to the powder surface polar components determined by IGC [(12)]. These charges influence the performance of dry powder inhalers and the regional lung deposition of the drugs [(13-14)]. It is clear from the above discussion the crucial effect of the surface components of protein powders on their inhalation formulations. Aging the surface during storage could change the surface energy and then change their tendency to aggregation in their formulations [(15)]. Many factors affect these changes during storage; critical is temperature. Many reports cited the impact of temperature on spray-dried solid state proteins [(16)]. The effects of storage temperature on the surface free energy of spray dried protein particles has, so far, not been investigated. In this paper, IGC will be used to detect these effects. Because the fragile protein conformation would be affected by storage temperatures, differential scanning calorimetry and biological activity tests will be also used to evaluate the integrity of lysozyme conformation after storage.

Material And Methods

Materials

Lysozyme white freeze dried powder, isolated from hen egg white, Biozyme laboratories.,UK, was used to prepare spray-dried lysozyme powders. Micrococcus Lysodeikticus was purchased from Sigma-Aldrich to measure the enzymatic activity.

Preparation of spray-dried lysozyme powders

Aqueous lysozyme solutions (5% w/v) were spray-dried through a Buchi 190 Mini, co-current two-fluid spray dryer. The feed solution and atomizing air pass separately to the atomized nozzle (0.5 mm diameter). The atomizing air is filtered through a 0.22 µm Milidisk filter (Millipore), heated to the required temperature, and moved to spray drying chamber using compressed air at 2-6 bar through the nozzle at flow rate 500-600 l/h. Silicone tubing and a peristaltic feed pump (1-100 rpm, Masterflex, Cole Parmer) were used to feed the protein solutions at a feed rate of 6.5 ml/min to the nozzle to be atomized and then dried by compressed hot air in the chamber. Water (room temperature) was circulated through a jacket around the nozzle to protect the protein against thermal denaturation. The dried powders were separated from the air stream by a cyclone separator and the air removed from the system by an aspirator. The outlet and inlet temperatures were monitored using two PT-100 temperature sensors. The lysozyme spray-dried samples were prepared at outlet temperatures of 65 ± 2 °C. The outlet temperatures were controlled by adjusting inlet temperatures to 130 ± 2.

Storage conditions

Spray-dried samples of lysozyme were covered by ParaFilm to prevent water egress and stored for one year at: 5 ◦C, room temperature and 40 ◦C. Samples were analysed at 0 and one year, i.e. pre and post-storage.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy of the spray dried powders was carried out using an Hitachi S-520 SEM (Hitachi, Tokyo, Japan). Powders were mounted onto a graphite layer on an aluminium stub. Then the mounted particles were coated with a thin layer of gold (ionized Aragon) under vacuum, using SEM tool sputter coated (Polarcon, Watford, UK).

Inverse Gas Chromatography

1.0 g of each powder was packed in glass columns with half loop (about 35 cm × 6 mm (outer diameter) × 4 mm (inner diameter)). Both dispersive and polar components of spray dried powders were measured using an automated Perkin Elmer Auto system series gas chromatography equipped with a flame ionization

detector (FID). Compressed air and hydrogen gas were used to fire the flame of FID. Nitrogen gas with a flow rate of 10 ml/min was used as a carrier for the injected probes. The column temperature was adjusted for all experiments to 30 °C. Four non polar probes, heptane (Sigma Aldrich, 99%+), octane (Aldrich-Chemical, 99%+), nonane (Aldrich- Chemical, 99%+) decane (Sigma Aldrich, 99%+), and also acidic polar probe chloroform ($CHCl_3$) (Laboratory reagent grade, 99%+), and basic polar probe tetrahydrofuran (THF) (Riedel-deHaën, 99%+) were injected inside the powder column at intervals of 30 minutes. Each probe was injected three times. Probe retention time was measured by the position of the peak maxima. Each column was analyzed twice.

The method of Schultz, et al., was used to calculate the surface components of the powders as follows.

$$RT \ln Vn = 2N (g_S^d)^{0.5} a (g_L^d)^{0.5} + \text{constant} \quad (1)$$

where N is Avogradro's number, R is the gas constant, T is the absolute temperature, and Vn is the net retention volume of n-alkane probes, g_L^d is the dispersive component of the surface free energy of the n-alkane probes and a is their interaction area.

The dispersive free surface energy g_S^d is then calculated from the slope of plotting $RT \ln Vn$ against $a (g_L^d)^{0.5}$.

From the alkane line, the specific free energy of adsorption ΔG_A^{sp} of polar probes can be calculated by subtracting the dispersive free adsorption energy of a polar probe (which equals to the dispersive free adsorption energy of a theoretical n-alkane reference having the same value of $a (g_L^d)^{0.5}$) from the total free surface energy of adsorption.

$$\Delta G_A^{sp} = -RT \ln(V_{ni} / V_n^{ref}) \quad (2)$$

where V_{ni} is the net retention volume of the polar probe and V_n^{ref} is the net retention volume of a theoretical n-alkane reference.

Determination of moisture content using Karl Fischer titration

Samples (in triplicate) were titrated using a 701 KF Titrino with 703 Ti stand (Metrohm Ltd., Switzerland) after calibration with a standard solution of water in methanol (5 mg water and 1 ml methanol).

Differential scanning calorimetry

Differential scanning calorimetry (DSC) thermograms were obtained using a 2920 Modulated DSC (Perkin Elmer). The temperature axis and cell constant of the DSC cell were calibrated with indium (10 mg, 99.999 % pure, melting point 156.60 °C, heat of fusion 28.40 J/g). Samples (3-8 mg) were weighed in aluminium pans on an analytical balance. The escape of water was facilitated by placing a pinhole in the lid prior to sealing. The samples were equilibrated at 25 °C and heated to 220 °C at a heating rate of 10 °C/min under continuous nitrogen flow. Each sample was analysed in triplicate.

Enzymatic assay

Lysozyme catalyzes the hydrolysis of β-1,4-glycosidic linkages of cell-wall mucopolysaccharides [17]. This principle has been used to measure the activity of lysozyme as follows. 30 µl of lysozyme solution 0.05 % in phosphate buffer 10 mM, Ph =5.2 was added to 2.97 ml substrate bacterial suspension 0.025 % of Micrococcus Lysodeikticus in phosphate buffer 66 mM, pH = 6.24. The decrease in the absorption at 450 nm was monitored by using a UV-Vis spectrophotometer (Pu 8700, Philips, UK). The activity has been determined by measuring the decrease in the substrate bacterial suspension concentration with time [18]. Hence the slope of the reduction in the absorption of light at 450 nm against the time at 3 min, starting when the protein solutions were mixed with the substrate bacterial suspension, was considered to be the indictor of the activity. All values were measured relative to the fresh spray dried sample. The measurements were performed in controlled temperature room at 20 °C to avoid fluctuation of lysozyme activity.

Results

Particle size

Scanning electron microscopy picture (Figure 1) shows that the lysozyme spray-dried particles were approximately spherical and hollow with a narrow size distribution. The average particle size was determined to be ~2-7 µm.

Surface free energy components

The dispersive and polar surface components of the fresh and stored samples are displayed in Table 1. Both the dispersive surface energy and the specific free energy of adsorption ΔG_A^{sp} of polar probes did not change significantly (t-Test: $P > 0.05$) after one year storage at 5 °C. However, the dispersive surface energy decreased significantly (t-Test: $P < 0.001$) by ~7 % and by ~12% after storage for one year at room temperature and at 40 °C, respectively. Moreover, the decrease in the polar components upon raising the storage temperature was more observable, as the ΔG_{sp} of $CHCl_3$ decreased significantly (ANOVA: $P < 0.001$) by ~15 % and 31 % after storage for one year at room temperature and at 40 °C, respectively. The

ΔG_{sp} of also THF decreased significantly (ANOVA: $P < 0.001$) by ~6 % and ~23 % after storage for one year at room temperature and at 40 °C, respectively. This means that the decrease in the adsorption free energy of the acidic probe was more than that of the basic probe, and so the surface basicity of lysozyme spray-dried powders decreased upon raising the storage temperature.

Karl Fischer titration and Differential scanning calorimetry

The DSC and Karl Fischer titration results, which are displayed in Table 2, shows that the water content (~6.6% w/w) and unfolding temperature (~ 201 °C) did not changes significantly (ANOVA: $P > 0.05$) during storage. No significant change (t-Test: $P > 0.05$) in the value of unfolding enthalpy after storage for one year at 5 °C was observed. However, for samples stored at higher temperature, a significant decrease (t-Test: $P < 0.001$)) in the unfolding enthalpy value of about ~12 % and ~21% after storage for one year at room temperature and at 40 °C, respectively, was observed. Figure 2 simplifies the data of the unfolding enthalpy.

Enzymatic assay

Although the above results of DSC illustrated that the extent of denaturation increased upon raising the storage temperature, no significant decrease was identified in the activity (ANOVA test: $P > 0.05$) (Table 3).

Discussion

Many factors control the size of spray dried particles, such as the properties of the atomized solutions (i.e. their viscosity, surface tension and concentration) and experimental conditions (i.e. outlet and inlet temperature, flow rate of feed solution and flow rate of drying air) [19]. The range of particle size of the prepared powders which was 2-7 µm has been found to be the best range to be inhaled (Louey, et al., 2004). This means that our optimized preparation conditions were ideal to prepare inhaled protein particles.

IGC has been used to measure the surface components of various non-protein pharmaceutical materials [20-21]. It has also been previously proved that IGC is a suitable tool to analyse the surface free energy components of non-protein spray-dried powders [22]. However, IGC has not been applied to the analysis of protein particles. Our results proved that the IGC was able to detect the changes in the surface free energy of the prepared spray dried lysozyme after storage. The surface basicity of the prepared powders can be explained as follows. During the spray drying, protein molecules with an amphoteric nature, tend to adsorb to hydrophobic/hydrophilic interfaces, e.g. the air/water interface. The adsorbed molecules, which are not replaced by the bulk molecules, prefer to adsorb with a flat orientation of their aromatic rings to the air/water interface [23]. Therefore exposure of these groups at the surface of particles would increase the dispersive surface free energy and the surface basicity, as their p electrons have the property of Lewis bases being able to form hydrogen bonds with acidic probes. Raising the storage temperature increased the number of unfolded molecules at the surface, which then aggregated due to the hydrophobic-hydrophobic interactions. This aggregation could happen preferably via hydrophobic regions rich with electrons, such as the aromatic rings of Tyr, Phe and Trp, due to their high van der Waals interactions since it is known that the interaction between the aromatic molecules is much stronger than the interaction between aliphatic molecules [24]. Consequently, upon increasing the storage temperature, the loss of the rich electron regions from the surface caused the decrease in the dispersive surface energy and surface basicity of the lysozyme spray dried powder surface, as these electrons participate in forming hydrogen bonds with acidic probes and give rise to high van der Waals interactions with all probes. While g_S^d and ΔG_{sp} of $CHCl_3$ would decrease due to a reduction in the electrons on the surface, ΔG_{sp} of THF would be reduced due to removal of some acidic groups from the surface.

The DSC results confirmed the formation of denatured aggregates in the samples stored at room temperature and the denaturation increased further at 40 °C. The decreased unfolding enthalpy is evidence of the unfolding of some lysozyme molecules during storage. Since the decrease in the values of unfolding enthalpy is considered to reflect the level of denaturation, because the enthalpy at the unfolding transition is a barrier energy which must be overcome to obtain the unfolded state (denatured state). The principle of aggregation progress is previously discussed by Arakawa [25].

The denaturation of lysozyme is reversible as no significant effect of storage on the enzymatic activities was noticed. This can probably be due to the re-naturation of denatured lysozyme molecules after separation from the aggregates upon rehydration, as the lysozyme concentration in the activity tested solutions, which were dilute, allowed the lysozyme in the denatured aggregates to separate and re-nature.

Conclusion

Protein powder should be stored at low temperature such as 5 °C to maintain their performance. Otherwise,

changes in their surface free energy occurs leading to potential alteration in their performance. It was found that the dispersive surface energy decreased significantly by ~7 % and by ~12% after storage for one year at room temperature and at 40 °C, respectively, with reduction in the surface basicity. The changes in surface energetics was due to protein denaturation as a significant decrease in the unfolding enthalpy value of about ~12 % and ~21% after storage for one year at room temperature and at 40 °C, respectively, was observed.

Tables

Table 1. The surface components of the fresh and stored spray-dried lysozyme powders.

Sample	g_S^d (mJ.m^{-2})	$-\Delta G_A^{sp}$ Chloroform (kJ.mol^{-1})	$-\Delta G_A^{sp}$ THF (kJ.mol^{-1})
Fresh sample	47.14 (1.38)	3.80 (0.04)	7.85 (0.16)
Stored at 5 °C	46.73 (.89)	3.76 (0.09)	7.93 (0.21)
Stored at Room	44.10 (0.34)	3.24 (0.07)	7.41 (0.05)
Stored at 40 °C	41.64 (0.36)	2.64 (0.20)	6.01 (0.23)

Values within parenthesis are S.D., n = 3.

Table 2. Denaturation temperature (Tm) and unfolding enthalpy (ΔH) determined by differential scanning calorimetry (DSC), and water content % w/w determined by Karl Fischer titration of the fresh and stored spray-dried lysozyme powders.

Sample	Water content % w/w	Tm °C	ΔH J/g
Fresh sample	6.8 (0.7)	201.4 (0.7)	7.83 (0.32)
Stored at 5 °C	6.5 (0.5)	201.3 (0.3)	7.79 (0.21)
Stored at Room	6.7 (0.3)	201.7 (0.1)	6.92 (0.24)
Stored at 40 °C	6.6 (0.8)	202.1 (0.8)	6.17 (0.19)

Values within parenthesis are S.D., n = 3.

Table 3. Enzymatic activity of the fresh and stored spray-dried lysozyme powders.

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Sample	% activity
Fresh sample	100.0 (2.8)
Stored at 5 °C	99.4 (2.4)
Stored at Room	98.7 (4.9)
Stored at 40 °C	97.6 (3.9)

Values within parenthesis are S.D., n = 3.

Figures

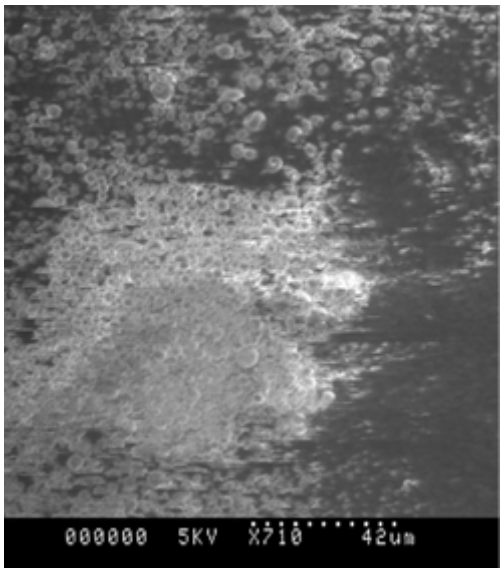

Figure 1. Scanning electron microscope picture of the spray dried lysozyme powder

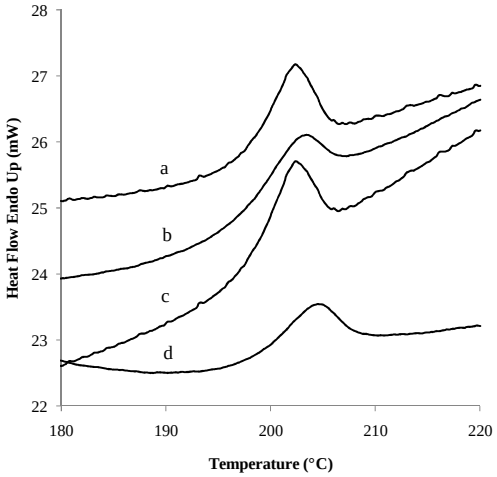

Figure 2. Normalised calorimetric data for the thermal denaturation of (a) Fresh spray dried lysozyme powder (b) Spray dried lysozyme powder stored at room temperature (c) Spray dried lysozyme powder stored at 5 °C (d) Spray dried lysozyme powder stored at 40 °C

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