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Research paper

Spray-drying of proteins: effects of sorbitol and trehalose on aggregation and FT-IR amide I spectrum of an immunoglobulin G

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Abstract

An immunoglobulin G (IgG) was spray-dried on a Büchi 190 laboratory spray-dryer at inlet and outlet air temperatures of 130 and 190 °C, respectively. The IgG solution contains initially 115 mg/ml IgG plus 50 mg/ml sorbitol. After dialysis, at least 80% of low molecular weight component was removed. After spray-drying the dialyzed IgG and immediate redissolution of the powder, an increase in aggregates from 1 to 17% occurred. A major shift towards increase β-sheet structure was detected in the spray-dried solid, which, however, reverted to native structure on redissolution of the powder. A correlation between aggregation determined by size exclusion chromatography and alterations in secondary structure determined by Fourier transformation infra-red spectroscopy could not therefore be established. On spray-drying a non-dialyzed, sorbitol-containing IgG only some 0.7% aggregates were formed. The sorbitol is therefore evidently able to stabilize partially the IgG during the process of spray-drying. Addition of trehalose to the liquid feed produced quantitatively the same stabilizing action on the IgG during spray-drying as did the sorbitol. This finding again points towards a water replacement stabilization mechanism. The IgG spray-dried powder prepared from the dialyzed liquid feed showed continued substantial aggregation on dry storage at 25 °C. This was substantially less in the non-dialyzed, sorbitol-containing spray-dried powder. Addition of trehalose to both dialyzed and non-dialyzed system produced substantial improvement in storage stability and reduction in aggregate formation in storage. The quantitative stabilizing effect of the trehalose was only slightly higher than that of the sorbitol. Taken together, these results indicate that both the sorbitol and trehalose stabilize the IgG primarily by a water replacement mechanism rather than by glassy immobilization. The relevance of this work is its questioning of the importance of the usually considered dominance of glassy stabilization of protein in dried systems of high glass transition temperature, such as trehalose. The low glass transition temperature sorbitol produces almost equal process and storage stability in this case.

Keywords: Immunoglobulin; Spray-drying; Stabilization; Sorbitol; Trehalose; Water replacement

1. Introduction

In the past 15 years, the spray-drying of therapeutic proteins has been successfully developed for the production of inhalable powders. Judicious selection of machine size and process conditions allows the preparation of protein-loaded powders having suitable aerodynamic properties and residual moisture content [1]. Spray-drying an aqueous solution of a pure protein produces aggregation [2] and/or loss of activity [3]. These instabilities can, however, be ameliorated, in some cases fully, by formulation measures. Disaccharides or surfactants in the liquid feed can prevent protein aggregation or inactivation during spray-drying [4]. The former also improve storage stability of the protein-loaded powder [3].

Spray-dried protein powders also have a potential application as a bulk storage material. If purified protein is insufficiently stable in aqueous solution, it is routinely freeze-dried to yield a storage stable powder. The most obvious disadvantage of this practice is the complexity of freeze-drying process control, which adds further cost to

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an already expensive process and cannot prevent occasional batch failure [5]. Spray-dried protein powders represent, therefore, a potential alternative to such lyophilized bulk protein. To be suitable as a bulk storage material, a spray-dried protein powder must have a particle size sufficiently large to ensure the flowability necessary for bulk powder handling (e.g. filling). A dry particle diameter of, say, ≥ 50 μm necessitates using a machine having adequate drying capacity and droplet/particle retention time [6]. A further major process requirement is a high powder yield from the spray-dryer. The remaining powder properties are, however, the same as those required for inhalable powders, viz. minimal protein damage during spray-drying, acceptably low residual moisture content, an overall spherical morphology, and satisfactory storage stability [7].

In this paper, we present the first part of our study of the spray-drying of an immunoglobulin G to produce powders suitable for bulk storage. The central aspect of the work presented here is an examination of how the aggregation status and secondary structural elements of the immunoglobulin G are affected by the spray-drying process and subsequent dry storage. The questions of optimization of particle size and powder yield are a scale-up matter and will be considered in a subsequent publication. The literature offers but few studies of spray-dried immunoglobulins. Costantino et al. [8] found increased aggregation of a recombinant humanized anti-IgE monoclonal antibody on spray-drying, which could be reduced on inclusion of either mannitol or trehalose in the liquid feed [9]. Although, mainly concerned with respirable powder properties, Platz et al. [10] found that spray-drying-induced aggregation of an immunoglobulin G could be reduced by addition of sucrose, mannitol or polyvinylpyrollidone. Neither of these authors considered, however, the relation between immunoglobulin aggregation and possible changes in secondary structure during spray-drying. Additionally, the differential effects of stabilizing adjuvents on process and storage stabilities of the spray-dried immunoglobulins were not sufficiently considered. In our study, we have examined these relationships and effects in a systematic fashion using an immunoglobulin G formulated with sorbitol in aqueous solution. Measurements of protein aggregation using size exclusion chromatography are accompanied by liquid and solid state Fourier transformation infra-red spectroscopy (FT-IR) to examine changes in the secondary structure of this predominately β-sheet protein during spray-drying and redissolution. A particular feature of this study is the comparison of sorbitol with trehalose as stabilizing agents for the immunoglobulin. This gives insight into the likely relative importance of 'water replacement' and 'glassy immobilization' [11] as potential stabilization mechanisms for the protein during droplet/particle drying within the drying tower and on subsequent dry storage.

2. Materials and methods

2.1. Materials

A human immunoglobulin G (IgG) was provided by Amgen as an aqueous solution (pH = 5.0) containing 115 mg/ml protein and 50 mg/ml sorbitol This solution was either used directly to prepare the liquid feed for spray-drying, or was first dialyzed to remove the most part of the sorbitol. In the latter case, approximately 3 ml was pipetted into a small dialysis bag of molecular weight cut-off 15,000. The filled bag was then immersed in 1 l of double-distilled water at room temperature and left overnight. After this treatment, the IgG solution (pH = 6.4) had approximately doubled in volume and was removed from the dialysis bag and immediately used to prepare the liquid feed for spray-drying. Repeated dialysis to remove completely the low molecular weight adjuvent was prohibited by the instability of the IgG in aqueous solution without sorbitol (see Section 3). The amount of sorbitol removed by this dialysis procedure was determined quantitatively by analyzing the amount of sorbitol present in the dialysate. The sorbitol was first oxidized to fructose by addition of nicotinamide-adenine-dinucleotide (NAD). The amount of reduced NADH formed in this reaction was then determined by titrating with iodine nitro tetrazolium chloride. The resulting formazon was measured by absorption at $\lambda = 492$ nm.

Analysis grade trehalose dihydrate was obtained from Sigma Chemicals (Munich), as were all further chemicals required for the analytical methods used here. Water was double-distilled from an all-glass apparatus.

2.2. Spray-drying procedure

Liquid feed was prepared from either the dialyzed or the non-dialyzed IgG solution. To this was added, as required, the appropriate amount of trehalose. The liquid feed was then immediately spray-dried on a Büchi Model 191 spray-dryer, which we used since only very limited amounts of the IgG were available. Recall that optimization of particle size and powder yield are not considered in this part of the work. The spray-drying process conditions were chosen to represent 'moderate' conditions based on our experience of spray-drying proteins with the Büchi spray-dryer: inlet air temperature, T_{in}, of 130 °C; outlet air temperature, T_{out}, of 90 °C; drying air flow rate, v_{da}, of 600 l/min; liquid feed flow rate, v_{lf}, of approximately 3 ml/min; and atomizing air flow rate, v_{aa}, of 700 l/h. In each experiment between 3 and 7 ml of liquid feed was spray-dried, containing 350–650 mg of total solids. Each spray-dried powder was removed from the Büchi's glass collecting vessel and transferred to small glass vials. Each was sealed under dry N_2 and then stored at 25 ± 0.5 °C.

2.3. Residual moisture content

Karl-Fischer titration was used to measure the residual moisture content of each spray-dried powder. Twenty milligram of powder was used for each determination, as fully described before [3].

2.4. Scanning electron microscopy (SEM)

Each spray-dried powder was examined on an Amray T100 scanning electron microscope after Au-sputtering.

2.5. Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) was used to characterize the aggregation status of the IgG A Bio Sep SEC S 3000 column was connected to Perkin Elmer HPLC comprising series 200 LC pumps, a UV detector, and a model ISS 200 auto sampler. The isocratic mobile phase comprised 20 mM sodium phosphate and 250 mM sodium chloride (pH 7.0) at a flow rate of 0.5 ml/min. Detection was performed at $\lambda = 280$ nm. Samples were prepared either directly from the liquid feed or by dissolving an appropriate amount of a spray-dried powder in a small volume of the mobile phase. Each sample injection of approximately 10 μl was chosen to contain approximately 40 μg of protein. The SEC system was calibrated daily using the standards apoferritin (MWt = 481 kDa) and albumin (MWt = 66 kDa) obtained from Sigma. Each chromatogram was integrated using the commercial Perkin Elmer Omniquant software.

2.6. Fourier transformation infra-red spectroscopy (FT-IR)

The FT-IR spectra of both liquid and solid samples were obtained using a Nicolet Magna IR 550 FT-IR spectrometer. Liquid samples containing 50 mg/ml IgG were examined in a thermostatted, specially constructed CaF_2 window of fixed sample layer thickness 5.8 μm [12]. Solid samples were produced by pressing KBr windows (1.5 mg protein + 200 mg KBr) on a Carver press at 7–8 T pressure. 250 spectra were recorded per sample at a sensitivity of 4 cm^{-1}. For the liquid samples, a water spectrum was first subtracted from the sample spectrum using the Nicolet software. All subsequent manipulations of the original spectra were performed using Galactic Grams software. The amide 1 band range between 1580 and 1720 cm^{-1} was first isolated from the complete (difference) spectrum using the 'Zap' option, and a Fourier de-convolution smoothing ($\gamma = 3$, 60% smoothing) then performed. Individual peak positions were identified from the second derivative spectrum of this range, before applying the 'Peak fitting' procedure to the original (difference) spectrum range to quantify the relative peak areas.

2.7. Differential scanning calorimetry

The glass transition temperature (T_g) of each powder yield by determined using a Mettler Toledo Model DSC 822 A 5–10 mg sample was examined in the temperature range 55–110 °C at a heating rate of 10 °C/min. T_g was calculated at the mid-point of the endothermic shift.

3. Results and discussion

All of the spray-drying runs gave the same deposition pattern of powder within the spray-dryer. The inside wall of the drying tower was clean, and only a small, powdery deposit of dry appearance was observed on the inside wall of the cyclone. The powder yield was removed from the glass collecting vessel and also the underside of its metal lid. The yields were all in the range 20–50%. The substantial losses were therefore, mainly caused by fine particles passing through the cyclone into the exhaust air.

3.1. Spray-dried, dialyzed IgG

The SEM of the spray-dried powder prepared from the dialyzed IgG liquid feed (Fig. 1a) shows the smooth particle surfaces typical of a spray-dried pure protein [1]. A substantial population of donut-shaped particles is present. The particle size range of 1–15 μm is typical for the Büchi and is a result of the high mass ratio of atomizing air to

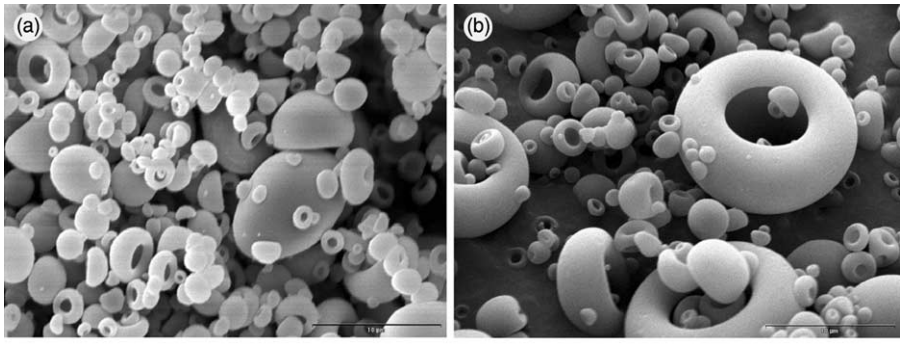

Fig. 1. Scanning electron micrographs (SEMs) of spray-dried powders. (a) dialyzed IgG; (b) non-dialyzed IgG.

liquid feed used with the two fluid nozzle (=approx. 3.3 [3]), causing generation of a very fine spray droplet size [13]. Larger, free-flowing particles of ≥ 50 μm diameter cannot indeed be prepared on the Büchi spray-dryer [1], and their preparation requires the use of a pilot-scale machine of substantially greater drying capacity [6].

Immediately after dialysis (but before spray-drying), the SEC of the IgG in solution (Fig. 2a) shows a dominating monomer peak at 167 kDa (99.3% from relative peak area) and 0.7% of an aggregate at 646 kDa (see Table 1).

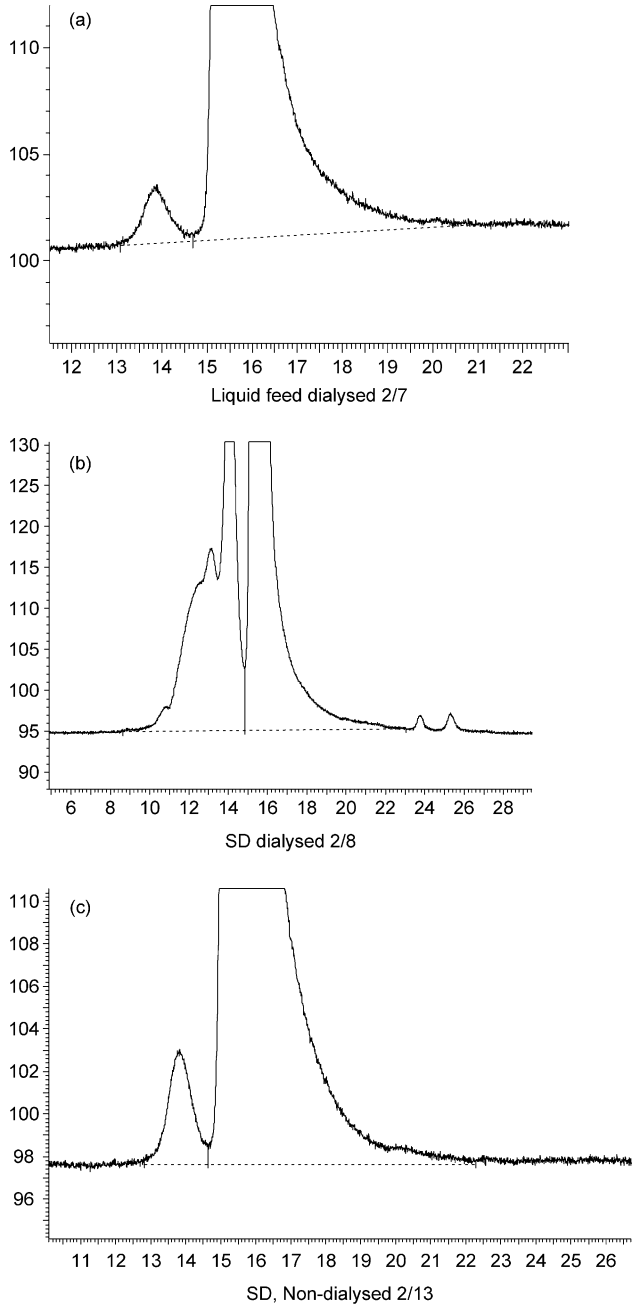

Fig. 2. Size exclusion chromatograms (SECs) of various IgG preparations. (a) dialyzed IgG used for liquid feed; (b) re-dissolved spray-dried powder from dialyzed IgG liquid feed; (c) re-dissolved spray-dried powder from non-dialyzed IgG liquid feed.

Table 1
Integrated peak areas from SECs for various IgG preparations

Figure #	Treatment	Peak #	RT (min)	MWt (kDa)	Peak area (%)
2a	Dialyzed liquid feed	1	13.9	646	0.70
		2	15.6	167	99.3
2b	SD dialyzed	1	10–14.8	–	17.3
	Re-dissolved powder	2	15.5	165	82.7
ns	Non-dialyzed liquid feed	1	13.8	673	0.50
		2	15.5	169	99.6
ns	SD non-dialyzed	1	13.8	610	1.2
	Re-dissolved powder	2	15.5	169	98.8

RT, retention time; SD, spray-dried; ns, not shown.

The result of analyzing the sorbitol content of the dialysate showed that the dialysis procedure removed $80 \pm 3\%$ ($n=3$) of the total sorbitol originally present. The dialyzed liquid feed (of volume 3 ml before dialysis, and 7.2 ml after) contained therefore 52 mg/ml total solids with a protein/sorbitol relative weight ratio of 11.5:1. Most, but not all, of the sorbitol had been removed. Spray-drying of this liquid feed at $T_{in}/T_{out}=130/85$ °C and subsequent immediate redissolution of the powder in water produces a major change in aggregation status of the IgG compared with the liquid feed. The SEC in Fig. 2b shows a broad aggregate band spread from the monomer peak at 165 kDa up to approx. 18 mDa. Superimposed on this band is a substantial aggregate peak centered at 537 kDa. Peak integration gives values of approximately 83% relative monomer and 17% total aggregates (see Table 1). The IgG suffers therefore, more aggregation on spray-drying and redissolution than that reported for other proteins. For example, spray-dried and re-dissolved pure trypsinogen lost 12% relative monomer at $T_{in}/T_{out}=130/90$ °C [2], accompanied by a 15% loss in enzymatic activity. Spray-dried and re-dissolved pure lysozyme showed a 13% loss in monomer at $T_{in}/T_{out}=120/85$ °C [14]. The IgG is also damaged more than was a spray-dried recombinant humanized anti-IgE monodonal antibody, which showed 12% loss of momoner at $T_{in}/T_{out}=90/60$ °C [8]. The substantial damage to the IgG occurs despite the presence of eight parts residual sorbitol to 92 parts protein in the solid. This relative amount of carbohydrate is, however, evidently too low to stabilize measurably the protein against aggregation on spray-drying, as also found, for example, with trypsinogen [2].

The damage to the dialyzed IgG on spray-drying is accompanied by changes in its FT-IR spectrum. Fig. 3a shows the second derivative difference spectrum of the amide I range, the original spectrum, and the curve-fitting result obtained for the dialyzed IgG liquid feed. The five major peaks in the second derivative spectrum lie at 1691, 1674, 1661, 1637, and 1615 cm^{-1}, and correspond closely with the five curve-fitted peaks in the original spectrum at 1697, 1669, 1664, 1638, and 1620 cm^{-1}. These peak

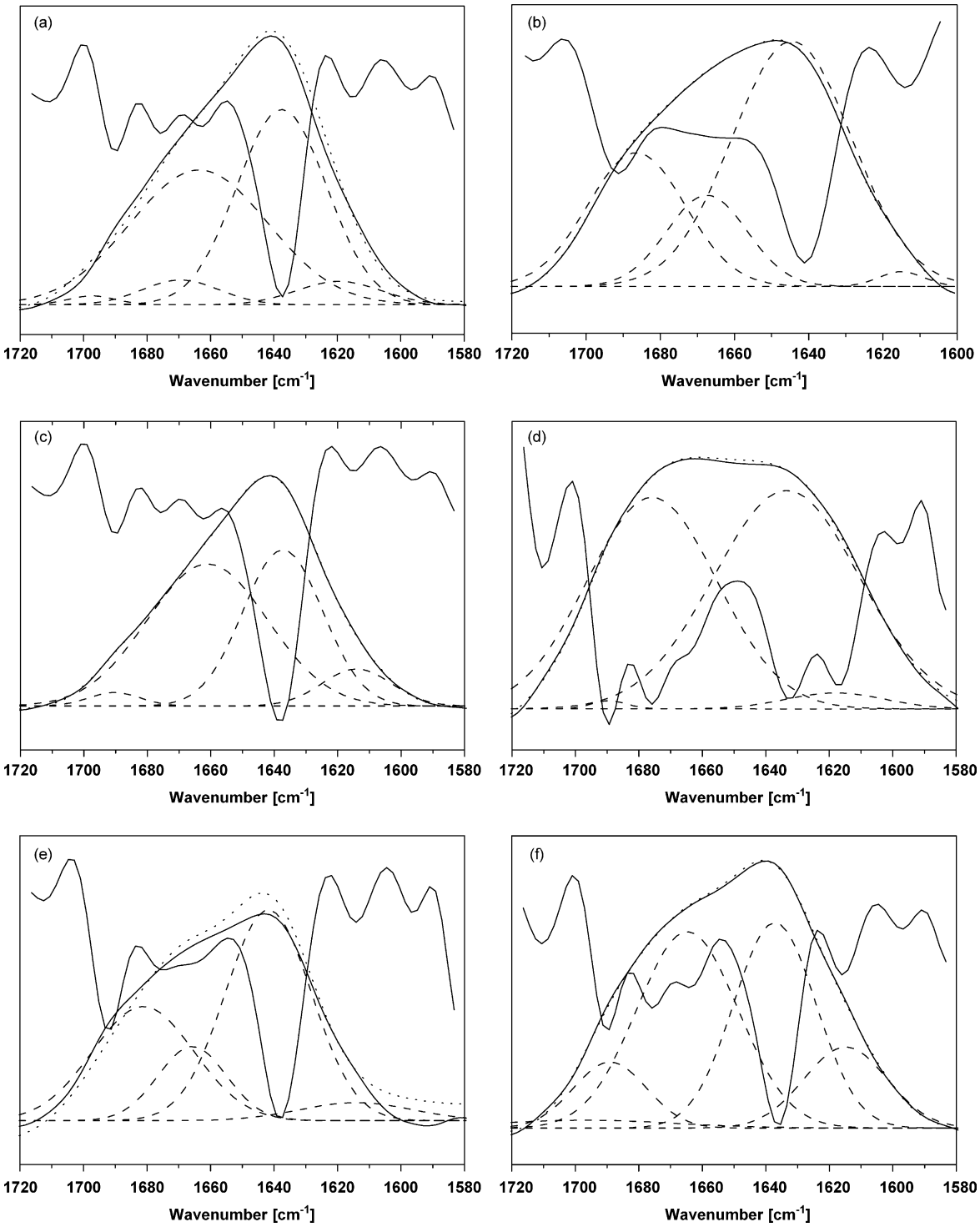

Fig. 3. Fourier transformation infra-red spectroscopy (FT-IR) difference spectra of amide I region of various IgG preparations. In each case, the second derivative spectrum is shown, as well as the original spectrum, and the result of the curve-fitting. (a) dialyzed IgG liquid feed; (b) spray-dried solid from dialyzed IgG liquid feed; (c) re-dissolved spray-dried solid from dialyzed IgG liquid feed; (d) non-dialyzed IgG liquid feed; (e) spray-dried solid from non-dialyzed IgG liquid feed; (f) re-dissolved spray-dried solid from non-dialyzed IgG liquid feed.

positions agree fairly well with those reported for a general IgG molecule by Fu et al. [15] at 1690, 1677, 1661, 1637, and 1615 cm^{-1}. We adopt Costantino et al.'s [16] assignation of these peaks as follows: β-sheet at 1697–1686, 1645–37, and 1638–1628 cm^{-1}; β-sheet or side chain at 1620–1615 cm^{-1}; turns at 1680–1676, and 1667–1660 cm^{-1}. Table 2 shows the quantitative results of the curve-fitting of the original difference spectrum according to these assignations. The dialyzed liquid feed is seen to have an IgG solution secondary structure of

Table 2
Results of curve-fitting of amide I bands obtained in FT-IR spectra for various IgG preparations

Treatment	Band (cm^{-1})	% Area	Assignation	Structures	Figure #
Dialyzed liquid feed	1697 (1691)	1.0	β-sheet	β-sheet 49.9%, other 50.7%	3a
	1669 (1674)	4.7	Turn		
	1664 (1661)	46	Turn		
	1638 (1637)	44	β-sheet		
	1620 (1615)	4.9	β-sheet		
SD dialyzed solid	1686 (1691	26	β-sheet	β-sheet 85.3%, other 15%	3b
	1667 (1666)	15	Turn		
	–	–	–		
	1645 (1641)	58	β-sheet		
	1615(1614)	1.3	β-sheet		
Re-dissolved SD dialyzed	1690 (1690)	2.1	β-sheet	β-sheet, 48% other 52%	3c
	1681 (1676)	0.1	Turn		
	1661 (1661)	52	Turn		
	1637(1638)	38	β-sheet		
	1614 (1614)	7.9	β-sheet		
Non-dialyzed liquid feed	1689 (1689)	0.3	β-sheet	β-sheet, 48% other 52%	3d
	1674 (1675)	52	Turn		
	1634(1632)	33	β-sheet		
	1618 (1617)	15	β-sheet		
SD non-dialyzed solid	1681 (1691)	31	β-sheet	β-sheet, 86% other 14%	3e
	1666 (1664)	14	Turn		
	–	–	–		
	1642 (1639)	49	β-sheet		
	1614 (1614)	5.5	β-sheet		
Re-dissolved SD non-dialyzed	1697 (1689)	2.3	β-sheet?	β-sheet, 60% other 40%	3f
	1689 (1675)	9.7	β-sheet		
	1664 (1662)	40	Turn		
	1637 (1635)	34	β-sheet		
	1614 (1615)	14	β-sheet		

The wave numbers given in brackets are those of the peak positions in the second derivative spectra. (SD, spray-dried).

approximately 50% β-sheet and 50% unordered (turns), a result obtained for three independently measured liquid feeds. Both Fu [15] and Costantino [16] quote an approximately 60% β-sheet content for IgG. The second derivative spectrum of the spray-dried solid (Fig. 3b) has lost the second turn band at approximately 1661 cm^{-1}. Consequently, the original spectrum curve-fitting now shows only two dominating β-sheet bands at 1686 and 1645 cm^{-1}, with the former greatly increased in magnitude compared with the corresponding 1687 cm^{-1} band of the liquid feed. The result is a secondary structure composition of 85% β-sheet and 15% unordered (Table 2). This substantial increase in β-sheet character of the IgG is therefore, a result of loss of the 1660–1667 cm^{-1} turn band, together with an increase in the 1686–1697 cm^{-1} β-sheet band. This finding contrasts with Costantino's claim that peak locations and relative areas of a spray-dried IgG were unchanged in the amide I range [16]. Although, Costantino's IgG was spray-dried under milder process conditions, viz. $T_{in}=90$ °C and $v_{Lf}=5$ ml/min, it is unlikely that this could account for such a substantial difference in the amide I spectra. Indeed, the reduction intensity of the 1654–1656 cm^{-1} α-helix band of spray-dried lysozyme was independent of T_{in} in the range 85–180 °C [14]. Griebenow and Klibanow [17] examined the freeze-drying of a number of proteins of various secondary structural composition and concluded that in all cases the β-sheet content increased substantially on drying. It is well established that the existence of intermolecular β-sheet elements in a polypeptide is favored in the solid state, since these can maintain a lower degree of solvation that other secondary structural elements such a α-helix turn or random coil [18]. We conclude therefore—in contrast to Costantino [16]—that this behavior is also shown by the IgG, despite its predominantly (≥50%) β-sheet composition in the native state.

On re-dissolving the IgG powder, the spray-drying-induced changes in the amide I spectrum seen in the solid all disappear. The second derivative spectrum (Fig. 3c) is now similar to that of the original liquid feed (cf. Fig. 3a). The turn band at 1661 cm^{-1} has reappeared. The peak fitting of the original spectrum (Fig. 3c) also shows reappearance of the strong turn band at 1661 cm^{-1}, accompanied by a large decrease in the β-sheet band at 1690 cm^{-1}. The result (Table 2) is a secondary structural composition of 48% β-sheet and 52% unordered (turn), which is the same as that found in the original dialyzed liquid feed. It is thus evident that the substantial perturbations of the IgG's 1686–1697 and 1660–1667 cm^{-1} bands induced by spray-drying are fully reversible, and are indeed lost on re-dissolving

the solid in water. This type of behavior has been noted before by Carpenter [18] for other proteins, and is taken as an indication of equivalent, reversible changes in the secondary structural elements of the IgG during spray-drying. Of interest is, however, the finding that the substantial change in aggregation status of the IgG induced by spray-drying and redissolution (cf. Fig. 2a and b) is not reflected in perturbations in the amide I region of the protein's FT-IR spectrum (cf. Fig. 3a and c). A relation between protein inactivation measured after re-dissolving and perturbation of the amide I spectrum in the solid state has been found with lysozyme [14], although the reversibility of the latter on redissolution of the spray-dried powder was not examined. We conclude that the formation of $>17\%$ aggregate in the re-dissolved, spray-dried powder is insufficient to alter the amide I spectrum of the solution. It follows that the substantial perturbation of the amide I spectrum seen in the spray-dried solid (Fig. 3b) must represent an increased β-sheet content of a large proportion, if not all, of the IgG molecules.

3.2. Spray-dried, non-dialyzed IgG

Recall that the non-dialyzed IgG solution contains 115 mg/ml IgG plus 50 mg/ml sorbitol. This is equivalent to a relative weight ratio of IgG/sorbitol of 2.3:1. The spray-dried particles prepared from this liquid feed (Fig. 1b) show the same smooth surfaces seen in Fig. 1a for the sorbitol-reduced, dialyzed system. These differ therefore from the typical highly wrinkled appearance of other spray-dried protein/carbohydrate mixtures, e.g. 5% w/w bovine serum albumin in trehalose [19]. The latter behavior is attributed to the protein molecules present at the water/air-interface of the atomized liquid feed, which increase the ratio of viscous to surface forces there and hence hinder smoothing of Marangoni fluctuations [20]. Since, IgGs are known to be surface active [21], the failure to observe a wrinkled surface appearance is evidently a result of the high weight fraction of protein in the solid.

The SEC of the liquid feed (not shown) is qualitatively identical to that in Fig. 2a for the dialyzed liquid feed, but shows less aggregate at 673 kDa (0.5%) than in the dialyzed solution (0.7%) (Table 1). This illustrates the sensitivity of the IgG to aggregation on removal of the sorbitol. Sorbitol is known to stabilize IgGs and hinder their aggregation in aqueous solution [22,23], attributed to a preferential exclusion mechanism [24]. After spray-drying, the SEC of the re-dissolved solid (Fig. 2c) shows 1.2% aggregate at 610 kDa with a corresponding monomer content of almost 99% (Table 1). The increase of 0.7% in aggregate on spray-drying and redissolution is therefore much less than the $>16.5\%$ increase in aggregation seen with the dialyzed IgG (Table 1). All of the IgG formulations examined here were fully X-ray amorphous after spray-drying (diffractograms not shown). The residual moisture contents of both the dialyzed and non-dialyzed IgG powders all lie in the range of 4.4–5.8% w/w (Table 3). The greatly improved stability of the non-dialyzed IgG to aggregation during spray-drying and redissolution is therefore a result of the substantial relative weight fraction of sorbitol in the solid (IgG/sorbitol = 2.3:1 ≡ 30% by weight sorbitol). It is known that sorbitol can stabilize proteins during drying processes. For example, it increased the enthalpy of denaturation at constant denaturation temperature of spray-dried egg white [25]. Also, a 1:1 weight ratio of sorbitol/protein reduced the process inactivation of catalase during freeze-drying from approximately 45% (pure, unprotected protein) to $<9\%$ [26].

Table 3
Residual moisture contents for various spray-dried formulations of the IgG

System	Dialyzed/non-dialyzed	% Water (w/w)
IgG	Non-dialyzed	4.4
IgG/treh (95:5)	Dialyzed	5.2
IgG/treh (90:10)	Dialyzed	4.5
IgG/treh (80:20)	Dialyzed	5.3
IgG/treh (90:10)	Non-dialyzed	5.8
IgG/treh (80:20)	Non-dialyzed	5.0

The FT-IR second derivative spectrum of the non-dialyzed liquid feed (Fig. 3d) shows only a single turn peak at 1674 cm^{-1} with a shoulder at approximately 1665 cm^{-1}, compared with the double turn peaks at 1664 and 1669 cm^{-1} for the dialyzed liquid feed (cf. Fig. 3a). Since, however, the combined areas of the double peaks equals that of the single peak, the curve-fitting from Fig. 3d yields 52% unordered (turn) and 48% β-sheet (Table 2). This is the same secondary structural composition as found with the dialyzed liquid feed (cf. Table 2). The measured increase in aggregate from 0.5 to 0.7% occurring on dialysis (Table 1) is therefore not accompanied by vital changes in the amide I spectrum. The second derivative and curve-fitted original spectra of the spray-dried, non-dialyzed IgG (Fig. 3e) show almost the identical result to that seen in Fig. 3b for the dialyzed system, i.e. an increase in β-sheet to $>85\%$ and a reduction in unordered to approx. 14% (Table 1). The 1681–1689 cm^{-1} β-sheet band increases in intensity at the expense of the 1664–1666 cm^{-1} turn band. On redissolution of the solid (Fig. 3f), this perturbation is, as observed with the dialyzed system, reversible (Table 1). The 1681–1689 cm^{-1} β-sheet band becomes smaller (although it does not return completely to its original very low value) and the 1664–1666 cm^{-1} turn band becomes larger (again, not to the original value of the 1674 cm^{-1} band). The substantial prevention of aggregation of the IgG by the sorbitol during spray-drying and redissolution is not therefore reflected by any alterations in the amide I spectrum of the protein compared with the sorbitol-reduced, dialyzed system. We conclude that aggregation of the IgG is not accompanied by changes in its secondary structure elements as determined by FT-IR. Although, this would agree with Costantino's [16] results, recall that he found no perturbations of the FT-IR spectrum in the spray-dried solid,

in contrast to our findings. The IgG shows a sharply increased content of β-sheet structure in the spray-dried solid, but this change is reversible on redissolution and not evidently related to the subsequent aggregation of the IgG detected in solution. Aggregation of the IgG occurs without change in its secondary structural elements as detectable from the amide I bands.

3.3. Storage stability of spray-dried IgG

The amount of total aggregates detected in the re-dissolved, spray-dried, dialyzed IgG increases sharply from 17% at $t=0$–37% after 12 months of dry storage at 25 °C (Fig. 4a). The poor spray-drying process stability of the unprotected (dialyzed) IgG seen above is therefore accompanied by substantial storage instability. This can be, however, greatly ameliorated by using the non-dialyzed IgG liquid feed containing IgG/sorbitol (2.6:1). Fig. 4b shows that in this spray-dried powder, the total aggregates increases from 1.2% at $t=0$–10% after 12 months of dry storage at 25 °C. This increase of approximately 8% in aggregates in less than half of the 20% increase observed with the dialyzed IgG. It follows that the sorbitol affects this substantial improvement in storage stability of the IgG in the spray-dried powder. As expected from the previous results, no changes in the amide I spectra of the solid or re-dissolved powder (dialyzed and non-dialyzed) could be detected on storage (spectra not shown). The enhanced spray-drying process stability can be intuitively attributed to a water replacement action of the sorbitol during drying of the droplets/particles in the drying tower. An improved storage stability would, however, be expected to be at least in part a result of glassy immobilization. Pure sorbitol has a glass transition temperature, T_g, of −7 °C in the fully dried state [27]. The T_g of the IgG/sorbitol (2.3:1) mixture is expected to be higher than this value because of the large weight fraction of protein present (≡60%), even accounting for the approximately 4.5% residual moisture (Table 3). The DSC scan showed, however, no thermal events in the temperature range −10 to +100 °C (result not shown). As we shall presently see in Section 3.4, a glass transition could be detected as an endothermic shift in the baseline for a non-dialyzed IgG/trehalose mixture of (80:20) at approximately +10 °C. Since, this solid contains IgG/sorbitol/trehalose in the weight fractions (2.3:1:0.8) and pure trehalose has a T_g of 115 °C [28], it follows that the T_g of the IgG/sorbitol (2.3:1) must be $<+10$ °C. As such, the spray-dried IgG/sorbitol (2.3:1) obtained from the non-dialyzed liquid feed was not in the glassy state on storage, and glassy immobilization cannot explain the improved storage stability of the non-dialyzed IgG over the dialyzed IgG. It is likely that a combination of water replacement and a diluting effect of the sorbitol present in the non-dialyzed IgG affects this improvement.

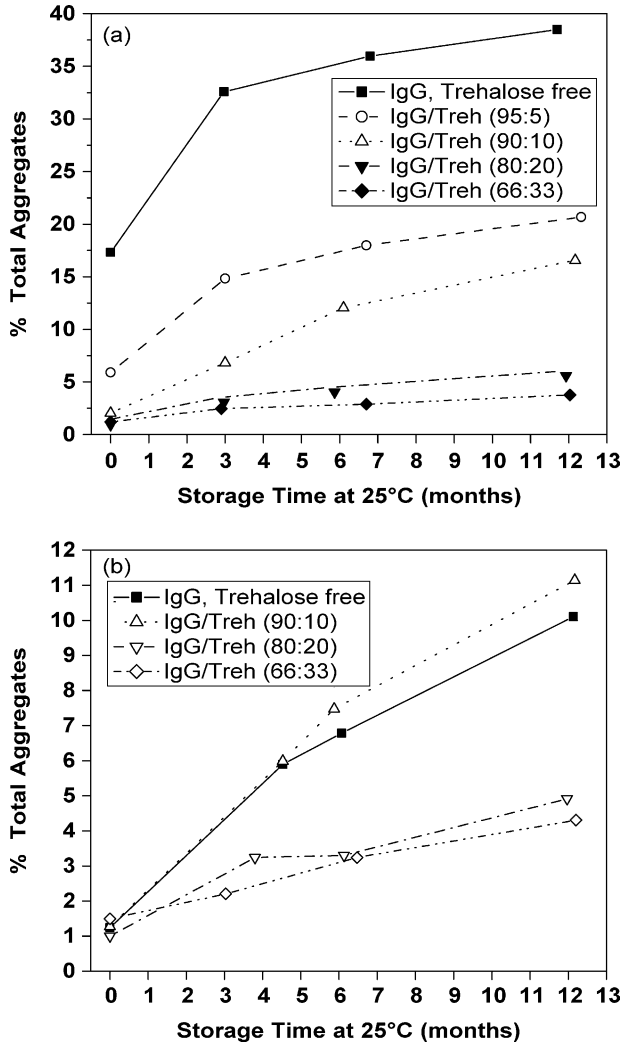

Fig. 4. Change in % total aggregates versus time for re-dissolved spray-dried IgG-containing powders. The powders were dry-stored at 25 °C. (a) dialyzed systems; (b) non-dialyzed systems. (■) pure IgG; (○) IgG/treh (95:5); (△) IgG/treh (90:10); (▼) IgG/treh (80:20); (◇, ◆) IgG (66:33).

3.4. Effects of added trehalose on spray-dried IgG

Fig. 4a and b also shows how the addition of trehalose to the liquid feed reduces the amount of total aggregates detected in the re-dissolved, spray-dried powders both immediately after spray-drying ($t=0$) and on dry storage at 25 °C. As the IgG/trehalose weight ratio decreases from (100:0) to (66:33), the broad aggregate band visible in the SEC of the pure, dialyzed, spray-dried IgG at $t=0$ (cf. Fig. 2b) progressively disappears, leaving only the monomer band at 165 kDa and a single, small aggregate band at 600 kDa (SECs not shown, for brevity). No effects of the added trehalose on the residual moisture contents of the spray-dried powders at $t=0$ are seen (Table 3). Fig. 5a gives a direct comparison of the stabilizing effects obtained with sorbitol and trehalose at $t=0$. Increasing amounts of added trehalose up to (80:20) to the dialyzed protein progressively reduce the amount of IgG aggregates formed during spray-drying and redissolution. In contrast, the addition of

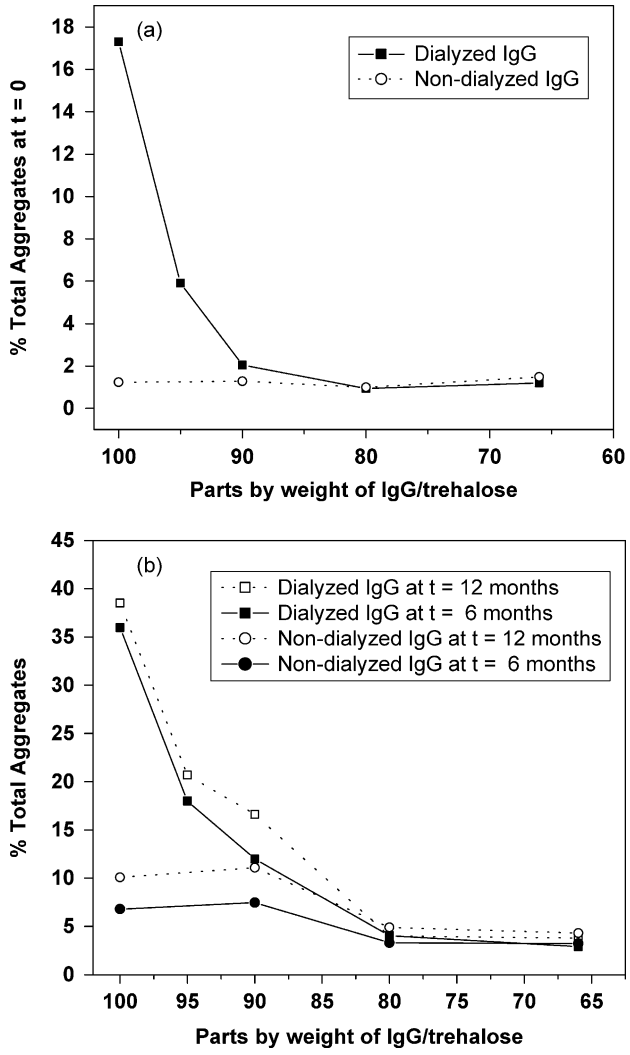

Fig. 5. Effect of added trehalose on relative % total aggregates in spray-dried IgG powders. (a) $t=0$ (immediately after spray-drying); (b) $t=6$ or 12 months of dry storage at 25 °C (□,■) dialyzed liquid feed; (○,●) non-dialyzed liquid feed. Data were taken from Fig. 4a and b.

trehalose to the non-dialyzed IgG has no measurable effect on aggregation at $t=0$. Recall that the dialyzed IgG/trehalose systems contain 8 wt % residual sorbitol not removed by the dialysis. The dialyzed IgG/trehalose (80:20) formulation reaches the same level of aggregation during spray-drying as that found with the trehalose-free, non-dialyzed IgG/sorbitol (2.3:1). On a weight basis (relevant for the T_g of binary mixtures [29]) 20% trehalose + 8% sorbitol stabilizes therefore, equally well as 30% sorbitol against IgG aggregation during spray-drying and redissolution. Allowing for the likely scatter in these results, the sorbitol evidently stabilizes the IgG equally well as the trehalose during the spray-drying process. The DSC scan of the dialyzed IgG/trehalose (80:20) showed a mid-point endothermic shift at 42.8 °C (not shown). The non-dialyzed IgG/trehalose (80:20) containing IgG/sorbitol/trehalose (2.3:1:0.8) had a T_g of 10 °C. Removal of the sorbitol from these mixtures therefore increases T_g from 10 to 42.8 °C. The maximum temperature experience by the droplets in the drying tower is the wet-bulb temperature, T_{wb}, which is approximately 45–50 °C at the process conditions used here. After passing the critical point of drying, the particle temperature will increase above T_{wb}, and, depending on the particle residence time in the drying tower, will approach T_{out} [30]. It follows from the similar stabilizing effects of trehalose and sorbitol that these carbohydrates cannot act by a glassy immobilization mechanism. If glassy immobilization was in any major way involved in stabilizing the IgG during the spray-drying process, then different degrees of stabilization would be expected for the non-dialyzed IgG/sorbitol (2.3:1) of $T_g < 10$ °C and the dialyzed IgG/trehalose (80:20) of $T_g = 42.8$ °C. Such are, however, not seen in Fig. 5a. Evidently water replacement is the essential stabilizing mechanism during droplet/particle passage through, and drying in the spray-dryer.

Trehalose similarly improves the storage stability of the dialyzed IgG at 25 °C in proportion to its relative weight ratio in the spray-dried solid (Fig. 4a). At an IgG/trehalose weight ratio of (80:20), an increase in total aggregates from 0.9% at $t=0$–5% after 12 months of dry storage is measured, compared with an increase for the unprotected, dialyzed IgG from 17% at $t=0$–38%. The trehalose also reduces aggregation on dry storage at 25 °C of the non-dialyzed spray-dried powders (Fig. 4b), although this is first noticeable with the (80:20) system and not the (90:10). Fig. 5b allows a quantitative comparison of the storage stabilizing efficacy of the sorbitol (in the non-dialyzed system) with that of the added trehalose (in the dialyzed system) on measured after 6 and 12 months of dry storage at 25 °C. As also seen at $t=0$ (cf. Fig. 5a), increasing trehalose in the liquid feed improves the storage stability of the dialyzed system, producing a continual decrease in % total aggregates after 6 and 12 months. The dialyzed IgG/trehalose (80:20) already has a lower level of aggregation after both storage times as that found with the trehalose-free, non-dialyzed IgG/sorbitol (2.3:1). Interpolation of the curves in Fig. 5b shows that the same level of aggregation formation, is reached at IgG/sorbitol = (85:15). Again on a weight basis, 15% trehalose + 8% residual sorbitol (not removed by the dialysis procedure) stabilizes the IgG during storage at 25 °C as well as 30% sorbitol (≡IgG/sorbitol (2.3:1)). Even allowing for experimental variation, the trehalose stabilizes the IgG during storage only marginally better than does the sorbitol. Since, both carbohydrates exert an equally strong water replacement effect on the process stability of the IgG (cf. Fig. 5a), this small difference in storage stabilizing efficacy must arise from another mechanism, e.g. glassy immobilization. The difference in stabilizing efficacy between trehalose and sorbitol is, however, very small and indicates that water replacement is also an important stabilizing mechanism during storage of the spray-dried composition. This is supported by the finding that addition of up to 10 weight parts of trehalose to

the non-dialyzed, sorbitol-containing system produces no improvement in storage stability (Fig. 5b). Here, the sorbitol is still the dominating factor producing better storage stability than that found in the dialyzed system. With ≤ 20 parts by weight trehalose, an improvement in stability is observed (Fig. 5b). Since both carbohydrates are equally good 'water replaces', this must be an effect of the better glass properties of the trehalose.

4. Conclusions

1. The dialyzed IgG examined here suffers major aggregation during spray-drying at $T_{in}/T_{out} = 130/85$ °C, and subsequent redissolution in water. This is not, however, reflected in any perturbations of the amide I IR-bands in the re-dissolved powder.
2. The amide I IR-bands of the spray-dried IgG solid show a distinct perturbation toward enhanced β-sheet secondary structure. The intensity of the 1690 cm^{-1} band (β-sheet) is enhanced at the cost of the 1661 cm^{-1} band (turn).
3. The presence of 30% by weight sorbitol (IgG/sorbitol = (2.3:1)) in the solid prepared from the non-dialyzed liquid feed substantially reduces aggregation of the IgG during spray-drying.
4. The addition of trehalose similarly protects the IgG during spray-drying. Twenty percent by weight trehalose (plus 8% residual sorbitol) in the solid (IgG/trehalose = (80:20)) produces the same reduction in aggregation as found with 30% by weight sorbitol (IgG/sorbitol = 2.6:1). Evidently, both sorbitol and trehalose stabilize the IgG during spray-drying by a water replacement mechanism.
5. Both sorbitol and trehalose improve the dry storage stability at 25 °C of the IgG by reducing aggregation. Trehalose is the slightly more effective storage stabilizer; 15% by weight trehalose (plus 8% residual sorbitol) gives the same stabilizing action as 30% by weight sorbitol. The stabilizing effect of these disaccharides on storage stability of the IgG is therefore likely a result of water replacement and the dilution effect, with a minor contribution by glassy immobilization.

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