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Effect of amino acids on the stability of spray freeze-dried immunoglobulin G in sugar-based matrices

Fakhrossadat Emami[a], Alireza Vatanara[a,*], Abdolhosein Rouholamini Najafabadi[a], Yejin Kim[b], Eun Ji Park[b], Soroush Sardari[c], Dong Hee Na[b,**]

[a] Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[b] College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea
[c] Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

The purpose of this study was to prepare spray freeze-dried particles of immunoglobulin G (IgG) using various combinations of trehalose and different amino acids (leucine, phenylalanine, arginine, cysteine, and glycine), and investigate the effect of the amino acids on the stability of IgG during the spray freeze-drying (SFD) process and storage. The morphology and structural integrity of the processed particles were evaluated by physical and spectroscopic techniques. SFD-processed IgG without any excipient resulted in the formation of aggregates corresponding to approximately 14% of IgG. In contrast, IgG formulations stabilized using an optimal level of leucine, phenylalanine, or glycine in the presence of trehalose displayed aggregates < 2.2%. In particular, phenylalanine combined with trehalose was most effective in stabilizing IgG against shear, freezing, and dehydration stresses during SFD. Arginine and cysteine were destabilizers displaying aggregation and fragmentation of IgG, respectively. Aggregation and fragmentation were evaluated by dynamic light scattering, ultraviolet spectrophotometry, size-exclusion chromatography, and microchip capillary gel electrophoresis. The IgG formulations prepared with leucine, phenylalanine, or glycine in the presence of trehalose showed good stability after storage at 40 °C and 75% relative humidity for 2 months. Thus, a combination of the excipients trehalose and uncharged, nonpolar amino acids appears effective for production of stable SFD IgG formulations.

1. Introduction

Therapeutic antibodies are the most rapidly growing category in the global pharmaceutical market. The annual sale of these drugs markedly increased from $27 billion in 2007 to almost $75 billion in 2013 (Ecker et al., 2015). Approximately 60 therapeutic antibodies including antibody-drug conjugates have been approved for the treatment of a wide range of diseases with a large number of candidates in clinical trials (Kim et al., 2016; Singh et al., 2017). Similarly to other therapeutic proteins, the large size, complex structure, and susceptibility of these antibodies to environmental stresses result in challenges to their physicochemical stability owing to several chemical and physical degradation factors (Lowe et al., 2011; Song et al., 2017). Hydrophobic surface interactions, pH, temperature, and environmental stresses have a significant impact on protein structure, where degradation products could be ineffective or less effective in comparison to the active subunit and in some instances, may be immunogenic (Daugherty and Mrsny, 2006; Zheng et al., 2017). Because most protein instabilities are water-mediated, dehydration of proteins can be a promising strategy for improving their stability because water removal can lead to the integration of protein into a solid matrix, which decreases protein mobility (Maltesen and van de Weert, 2008; Depreter et al., 2013).

Several techniques including freeze drying, spray drying, spray freeze-drying (SFD), vacuum drying, and foam drying have been used for drying and particle design of proteins (Maa and Sellers, 2005; Heljo et al., 2013; Kanojia et al., 2016; Lim et al., 2016). Among these technologies, SFD is an improved drying method to process antibodies because of the high specific surface area of dried powders leading to fast reconstitution time and its capacities for particle engineering (Wanning et al., 2015). During the SFD process, a solution is sprayed, frozen in liquid nitrogen, and subsequently lyophilized. In this process, proteins may experience shear stress at the air-liquid interface, freezing stress, and dehydration stress, which can result in structural perturbation (Saluja et al., 2010). Conventionally, stabilizers and cryoprotectants have been added to overcome these stresses and improve protein stability (Kanojia et al., 2016). However, no stabilizer can guarantee the

* Correspondence to: A. Vatanara, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
** Correspondence to: D.H. Na, College of Pharmacy, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul 06974, Republic of Korea.
E-mail addresses: vatanara@tums.ac.ir (A. Vatanara), dhna@cau.ac.kr (D.H. Na).

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stability of all proteins. Thus, the formulation of antibodies using an appropriate combination of excipients is challenging (Kheddo et al., 2014). A series of additives including sugars, amino acids, and biopolymers have been reported to have protective effects through water replacement, immobilization of proteins in a solid matrix, and retention of the proteins in an amorphous state, with high glass transition temperature (T_g) (Depreter et al., 2013; Tonnis et al., 2014; Kanojia et al., 2016). Among sugars, trehalose has a high T_g and has been widely used as a lyoprotectant for several proteins (Adler et al., 2000; Tonnis et al., 2014; Lim et al., 2016). Amino acids are also well-known excipients in stable protein formulations (Faghihi et al., 2014). The amino acids arginine, glycine, and histidine are used as stabilizing excipients in commercial protein therapeutics (Ajmera and Scherließ, 2014).

Although the capabilities of the SFD process have been studied for several biopharmaceuticals (Maa et al., 1999; Yu et al., 2006; Saluja et al., 2010; Kanojia et al., 2016; Poursina et al., 2016), there has not been any systematic investigation on the effects of this process on the structure of immunoglobulin G (IgG) as a typical model antibody. In this study, IgG was processed by SFD and the effects of trehalose and/or different amino acids as excipients on the physicochemical stability of IgG were evaluated by various analytical methods. Five amino acids with hydrophobic (leucine, phenylalanine), hydrophilic (arginine), polar sulfur-containing (cysteine), or nonpolar (glycine) side chains were used in combination with trehalose (Faghihi et al., 2014). They represent different physicochemical properties as excipients. The structural stability of IgG in formulations during SFD was evaluated by circular dichroism, fluorescence spectroscopy, X-ray diffraction, and ultraviolet spectrophotometry. The effect of the different amino acids on the physicochemical stability of IgG formulations in the trehalose matrix during SFD and after storage at 40 °C for 2 months was assessed by size-exclusion chromatography and microchip capillary gel electrophoresis.

2. Materials and Methods

2.1. Materials

Human intravenous polyclonal IgG derived from pooled plasma was obtained from Kedrion (Barga, Italy). 8-Anilino-1-naphthalene sulfonic acid ammonium salt (ANS), D-(+)-trehalose dihydrate, leucine (Leu), cysteine (Cys), phenylalanine (Phe), arginine (Arg), and glycine (Gly) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Protein 230 Labchip kit including chips and reagents were supplied by Agilent Technologies (Waldbronn, Germany). All other reagents, unless indicated otherwise, were purchased from Sigma-Aldrich and were used as received.

2.2. Spray Freeze Drying of IgG

Spray freeze-dried formulations of IgG containing trehalose and/or the different amino acids (Leu, Phe, Arg, Cys, and Gly) were prepared according to Table 1. The feeding solutions were prepared by adding 200 mg of trehalose (2%) and/or 25 or 50 mg of amino acids (0.25 or 0.50%) to 2% (w/v) IgG solutions. All samples were filtered through 0.22 μm membrane filter prior to SFD experiment. In each experiment, 400 ml of liquid nitrogen was poured into a glass container to produce a cryogenic vapor and the feeding solution was sprayed through a two-fluid nozzle into a vapor above liquid nitrogen at a feeding rate at 6 ml/min using Büchi 191 spray dryer (Flawil, Switzerland) as described previously (Poursina et al., 2016). The formed slurry was left on a bench until the excess nitrogen evaporated and then, the frozen droplets were transferred to a lyophilizer (Christ, Germany). Primary drying was carried out during the first 24 h at −50 °C and 0.005 mbar. Secondary drying was done by increasing the temperature to +10 °C during one day. The dried particulate powders were removed from glass container and stored at 4 °C until use.

Table 1
Formulations of IgG prepared by SFD.

Formulations	IgG	Trehalose	Amino acids
F_1	200 mg	–	–
F_2	200 mg	200 mg	–
F_3	200 mg	200 mg	Leu 25 mg
F_4	200 mg	200 mg	Leu 50 mg
F_5	200 mg	200 mg	Phe 25 mg
F_6	200 mg	200 mg	Phe 50 mg
F_7	200 mg	200 mg	Arg 25 mg
F_8	200 mg	200 mg	Arg 50 mg
F_9	200 mg	200 mg	Cys 25 mg
F_{10}	200 mg	200 mg	Cys 50 mg
F_{11}	200 mg	200 mg	Gly 25 mg
F_{12}	200 mg	200 mg	Gly 50 mg

2.3. Field Emission-scanning Electron Microscopy (FE-SEM)

FE-SEM was performed using a model S-4160 microscope (Hitachi, Tokyo, Japan) to evaluate the size and morphology of the tailored powders. The powder samples were each mounted on an aluminum stub using double-sided adhesive tape and coated with a thin layer of gold by Technics Hummer-II sputter coater. The accelerating voltage was set at 30 kV.

2.4. Differential Scanning Calorimetry (DSC)

The thermal behavior of the SFD samples was evaluated by DSC (Mettler Toledo, Greifensee, Switzerland). A small amount of each powder (5–10 mg) was placed in an aluminum pan and the pan was sealed. Thermograms were recorded at a scanning rate of 10 °C/min over a temperature range of −20 to 300 °C.

2.5. X-ray Diffraction (XRD)

The amorphous/crystalline patterns of pure trehalose, Leu, Phe, Arg, Cys, Gly, and SFD powders were obtained by XRD using an X'pert PRO MPD diffractometer (PANalytical, Almelo, Netherlands). Ni-filtered CuKα-radiation scattered in the crystalline parts of the samples was acquired at room temperature over a diffraction angle (2θ). The sample was scanned between 10° and 60° with a scanning speed of 5°/min, operating at a tube load of 40 kV and 30 mA.

2.6. Circular Dichroism (CD)

CD spectra were recorded in the range of 190–250 nm using a J-1500 spectropolarimeter (Jasco, Tokyo, Japan) with quartz cell path length of 0.1 mm at 20 °C. IgG and formulation samples at 1 mg/ml in 10 mM phosphate buffer (pH 7.4) were filtered using a syringe filter with 0.45 μm pore size. Spectra were obtained at a scanning rate of 200 nm/min and wavelength steps of 0.5 nm, with five scans accumulated. The spectrum of the buffer/additives mixture was subtracted from the sample spectra. By assuming 113 Da as average weight of amino acid residue in IgG, mean residue ellipticity (Kelly et al., 2005) and content (%) of secondary structure elements was calculated using the spectropolarimeter software (JWSSE-513 protein secondary structural analysis program).

2.7. Fluorescence Spectroscopy

Extrinsic fluorescence spectra of the protein samples were monitored by top reading using Infinite™ M200 PRO microplate readers (Tecan, Msing Inf, Switzerland). Approximately 190 μl of diluted IgG formulation was added to 10 μl of 20 μM ANS stock solution to produce a final dye concentration of 1 μM. From this mixture, 200 μl was loaded into wells of a 96-well polypropylene plate in darkness. Spectra