Stability Assessment of Biopharmaceutical Formulations

Joana Baltazar Domingues

Abstract: Antibodies are widely used for therapeutic purposes and immunochemical and biochemical analyses. A common problem associated with therapeutic antibodies is to ensure their long term stability which constitutes a formulation challenge. The stability of antibodies has been studied in the absence and in the presence of common additives present in therapeutic formulations in order to identify which additive leads to higher antibody stabilization. First, IgG stability was evaluated against heat denaturation, pH induced denaturation and mechanical shear stress induced degradation. Then, IgG stability in solutions with sucrose, glycine, maltose, L-histidine and D-trehalose was tested against thermal and pH induced denaturation. Aggregation studies were also conducted for different values of temperature and pH using dynamic light scattering. The percentage of retained biological activity was determined by affinity chromatography and changes in IgG's secondary structure were assessed by circular dichroism spectroscopy.

IgG was found to be resistant to shear stress up to 49 Pa induced in a concentric-cylinder shear device and against induced denaturation at pH 3. At pH 2, irreversible denaturation occurred at some extent. Regarding heat denaturation, IgG suffered only mild structural changes at 60°C but at 70°C significant loss of biological activity, extensive structural changes and IgG precipitation were verified. IgG showed higher tendency for aggregation upon incubation at 70°C than upon incubation at pH values distant from the isoelectric range (pI ≈9).

Amongst the additives studied, maltose was identified as the best IgG stabilizer since it provided complete stabilization at low pH and the highest stabilization against heat denaturation. Generally, maltose was also able to reduce IgG's tendency for aggregation.

***Keywords**:* Antibodies, Stability, Aggregation, Maltose, Circular Dichroism Spectroscopy, Dynamic Light Scattering

Abreviations: mAb, monoclonal antibody; IgG, immunoglobulin G; CD, circular dichroism; DLS, dynamic light scattering; PBS, phosphate buffer saline; pI, isoelectric point; CCSD, concentric-cylinder shear device

1. INTRODUCTION

Therapies with biotechnology-derived products have been increasing steadily over the past years. Antibodies, in particular, are widely used as convenient and valuable tools not only for immunochemical and biochemical analyses but as well as for therapy purposes. Progress in monoclonal antibody technology has led to the production of substantial amounts of highly specific monoclonal antibodies (mAbs) (1). In fact, mAbs are the fastest growing class of biopharmaceuticals (2) with over 25 mAbs already approved for several diseases. The sales volume of mAbs is expected to reach at least 70 billion US Dollars by 2015 (3).

IgG is a multi-domain protein with domains structurally independent, compact globular regions consisting of continuous stretches of the polypeptide chain approximately 100 amino acids long, with a characteristic fold, containing two β-sheets and essentially no α-helices. These proteins are composed of four polypeptide chains connected by disulphide bonds and non covalent forces. The four polypeptide chains are grouped together in different fragments, two identical Fab segments and one Fc segment, thus forming a Y-shaped conformation. The antigen binding sites are located on the far ends of the Fab segments. The Fab segments are linked to the Fc by the hinge region, which varies in length and flexibility in the different antibody classes and isotypes. Both Fab and Fc fragments consist of four of the above mentioned globular regions. (4).

The presence of different domains in immunoglobulins has a strong impact on their overall behavior. It has been suggested that, at least to some extent, the domains denature independently and that changes in the solution conditions may affect the different domains differently. Vermeer *et al.* (2000) showed that IgG has two main transitions that are a superimposition of distinct denaturation steps. The transitions were independent and the unfolding was followed by an irreversible aggregation step. Each transition had different sensibility to changes in temperature and pH (4). Vermeer *et al*. (1998) had previously shown that heat-induced denaturation doesn't lead to complete unfolding into an extended polypeptide chain, but leaves a significant part of the IgG molecule in a globular or corpuscular form. It was also verified that

heating dissolved IgG causes a decrease of the fractions of β-sheet and β-turn conformations, whereas those of random coil and, to a lesser extent, α-helix increased (1). Wefle *et al.* (1999) observed conformational changes and the formation of new structures at pH values between 3.5 and 2.0 (5). Szenci *et al.* (2005) observed that IgG's conformational stability decreases with decreasing pH while the resistance to aggregation increases (6).

Immunoglobulins have the tendency to form aggregates, especially when present in solution in high concentration, which is often needed to reach the intended therapeutic effect (7). Both native and denatured proteins may form aggregates: aggregation of denatured proteins is extremely common while aggregation of native proteins is most common upon long periods of storage. Aggregation is undesirable because it causes a decrease in drug efficacy and potential problems in drug administration (8). Therefore, it is essential to monitor aggregation as part of the quality control process.

In order to guarantee the effectiveness and quality of therapeutic antibodies it is essential to ensure the stabilization of the active-product, not only during production and purification, but also during storage. Thus, there is the need to find proper formulations for those products, which can be challenging on its own. The successful formulation of proteins depends on a thorough understanding of their biological and physico-chemical characteristics since the therapeutic activity of proteins is highly dependent on their conformational structure, which is, on its turn, flexible and sensitive to external conditions (9).

The scope of this work was to assess the stability of different formulations of human therapeutic antibodies against temperature induced denaturation, pH induced denaturation and mechanical shear stress in order to identify which of the additives commonly present in these formulations lead to higher antibody stabilization.

2. MATERIALS AND METHODS

2.1 Materials

Human IgG for therapeutic use (with the commercial name Gammanorm) was acquired from Octapharma as a solution with a nominal concentration of 165mg/mL and a purity degree of 95%.

Glycine (molecular weight of 77.05 Da), D-trehalose dehydrate (molecular weight of 378.33 Da) and sucrose (molecular weight of 342.31 Da) were purchased from Sigma. L-histidine with a molecular weight of 115.15 Da was acquired from Sigma-Aldrich. Maltose monohydrate monocrystalline (molecular weight of 360.32 Da) was purchased from Merck. The remaining chemicals were of analytical grade.

2.2 Samples Preparation and Incubation

IgG solutions with the concentrations of 1, 5, 10 and 15 g/L were prepared. For the experiments without additives the preparation was done with PBS (10 mM phosphate, 150 mM NaCl, pH ≈ 7.4). For the second set of experiments samples were prepared with PBS with each additive added individually in concentrations of 10% (w/v) – for Glycine, Maltose monohydrate crystalline, Sucrose and D-trehalose dihydrate – and 2% (w/v) – for Maltose monohydrate crystalline and L-histidine.

Thermal induced denaturation

The thermal stability was assessed by incubation at 60°C for 50 hours and at 70°C for 8 hours. In the presence of each additive, the incubation took place for 8 hours at 70°C. Since the objective was to study the irreversible heat denaturation of IgG, the samples collected were then stored at 4°C. All the samples were diluted 5 times with PBS (pH 7.4) before further analysis.

pH induced denaturation

pH induced denaturation was evaluated by incubation of IgG at pH 3.02 and 2.1, respectively for 50 hours and 9 hours. In the presence of the additives, IgG was incubated at pH 2.1 for 9 hours. In all situations, the incubation samples were kept at 4°C to eliminate any possible any temperature induced denaturation effect. In the incubation assays at pH 2.1, 5 µL of NaOH 1M were added to restore the pH back to approximately 7 in order to stop the denaturation process and to allow further analysis. All the samples were diluted 5 times with PBS (pH 7.4) before further analysis.

CCSD assay

The denaturation induced by shear stress was evaluated in a vorticular flow filtering system, Benchmark Gx from Membrex (Garfield, N.J., USA) without a filtration membrane – CCSD (concentric-cylinder shear device).

The recirculation rate of the IgG solutions (100 mL) through the annulus was 116.4 mL/min, with a corresponding superficial velocity of 0.003 m/s. The recirculation of the solutions was accomplished with a peristaltic pump (from Watson-Marlow, Massachussets, USA).The operating velocities of the inner cylinder of the CCDS were 1000, 2000 and 3000 rpm. Two initial

concentrations of IgG were tested: 1 g/L – tested at the 1000, 2000 and 3000 rpm - and 15 g/L – tested only at 3000 rpm.

2.3 Affinity Chromatography

The protein quantification was conducted in an Äkta 10 System (GE Healthcare, UK) with an analytical POROS Protein A Affinity Column and/or an analytical POROS Protein G Affinity Column both from Applied Biosystems (Foster City, CA, USA). IgG adsorption to the column was performed in PBS. Elution was accomplished with the desorption buffer (12 mM HCl, 150 mM NaCl, pH 2-3). After analysis the column was re-equilibrated with the storage buffer (10 mM NaH_2PO_4, 0.02% NaN_3, pH ≈ 7.4). Absorbance was measured at 280 nm and IgG concentration estimated based on a calibration curve prepared by dilution of a 1 g/L stock solution of IgG.

The results are presented in the form of the percentage of retained biological activity, which represents the fraction of the initial IgG molecules that remain biologically active at a given time (ratio between the concentration of IgG in the sample under analysis and in the initial sample).

2.4 Circular Dichroism

The CD spectra were measured with an Applied Photophysics spectropolarimeter, model PiStar - 180 (Leatherhead, UK). For the far-UV measurements (200-250 nm), a Quartz cuvette (Hellma, USA) with a light path of 1 mm was used. The antibody concentration in the measured samples was of 100 mg/L, except in the glycine and maltose at pH 2.1 assays, in which it was 80 mg/L. The CD spectra obtained are the result of the average of 10 to 20 scans. The monochromator bandwidth was set to 2nm and each measurement done with a 1 nm step. A blank was performed with the corresponding control buffer. A tool from the Pro-Data Analysis software was used to smooth the curves by a parameter of 10.

The mean residue ellipticity was determined using a mean residue weight of 113.16 Da (10).

2.5 Dynamic Light Scattering

The particle size was determined by DLS using a Malvern Zetasizer nano- ZS (Malvern Instruments Ltd, Worcestershire, UK) with a 5 mW He-Ne laser (633 nm) and a fixed scattering angle of 173 degrees. The direct measurements of the particle size were performed at a constant temperature of 25°C. The IgG concentrations studied in this assay were of 1 and 15 g/L.

For the measurements 1.5 mL disposable polystyrene cuvettes were used. The number of scans was determined by the equipment according to the characteristics of each sample. The results obtained were processed and analyzed with the Zetasizer nano-ZS software.

3. RESULTS AND DISCUSSION

3.1 Stability assessment of IgG without additives

3.1.1 Thermal induced denaturation

The thermal stability of IgG was assessed at both 60 and 70°C.

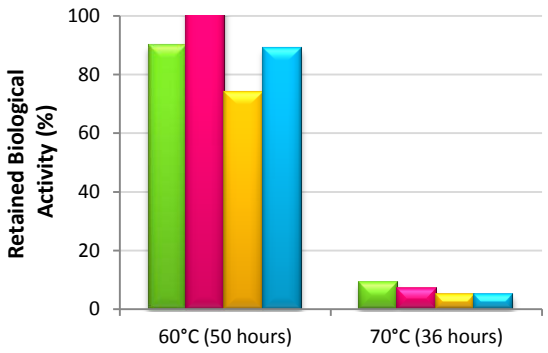

Figure 1 – Retained Biological Activity (%) after of incubation at 60°C for 50 hours and at 70°C for 36 hours of IgG solutions in PBS (pH 7.4) with the initial concentrations of 1 g/L (■), 5 g/L (■), 10 g/L (■) and 15 g/L (■).

After 50 hours of incubation at 60°C there were no major losses of biological activity (Figure 1). In contrast, upon incubation at 70°C for 36 hours denaturation occurred at a much higher extent. The major loss of biological activity is almost immediate and occurs between 0 and 2 hours of incubation at 70°C. After 8 hours of incubation the biological activity is almost the same as after 36 hours and, like that, this was the time adopted for further comparison.

After incubation at 70°C it was observed IgG precipitation. IgG unfolds as a consequence of the heat treatment, causing the exposure of the hydrophobic residues located in the interior of the protein (17), which triggers the formation of aggregates through intermolecular hydrophobic binding (16). The aggregates result from the partial unfolding of the Fab-fragments, which then become associated leaving the Fc fragments exposed to the aqueous environment (1), (16). The formation of these aggregates result in the observed precipitation of the IgG molecules. It has been demonstrated that the heating process of IgG doesn't

lead to a complete unfolding into an extended polypeptide chain, which occurs because the rate of aggregation is so fast that the molecules become locked in the aggregates formed before being completely denatured. The aggregation step is thus the step that induces the irreversibility of the heating denaturation process (reversible unfolding step, followed by an irreversible process that locks the unfolded protein in a state from which it can no longer refold) (16).

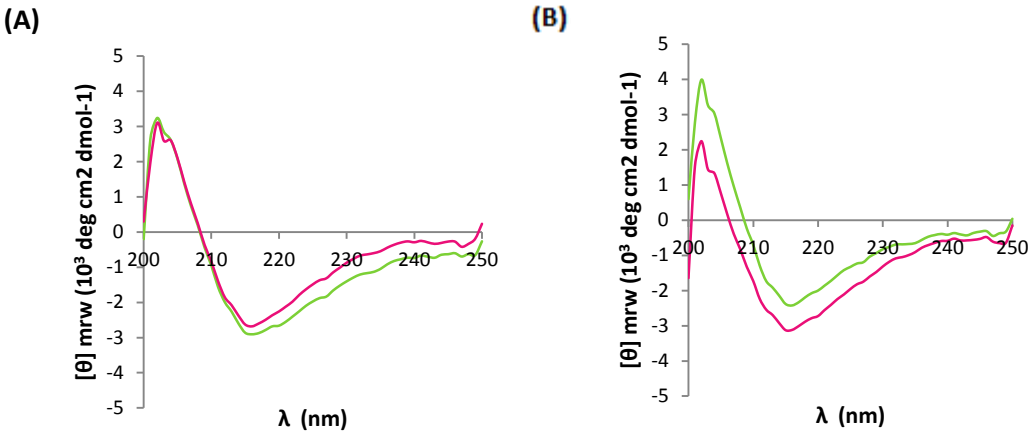

Figure 2 - Far-UV CD spectra of IgG in PBS (pH 7.4) before (—) and after 50 hours (—) of incubation at 60°C for the initial concentration of (A) 1 g/L and (B) 15 g/L.

The changes in the secondary structure were evaluated by CD spectroscopy. It is observable both in Figure 2A-B and Figure 3A-B that the CD spectra of intact IgG (before incubation) are that of a typical immunoglobulin, with a minimum at approximately 217nm, a maximum close to 200 nm and zero intensity around 207 nm, thus representing a high content of β-sheet. Upon incubation at 60°C the general shape of the curve remains similar to the typical β-sheet curve for both samples, even though that, for 15 g/L, the minimum at 217 nm broadens and shifts to a lower wavelength. The wavelength corresponding to zero intensity also shifts to a lower value and an overall decrease in the ellipticity occurs. Nevertheless, the changes in the secondary structure weren't significant. The samples after 8 hours of incubation at 70°C present a curve with only negative contributions and generally close to zero, similar to the typical spectrum of a random coil motif. Furthermore, the ellipticity near 206.5 nm is negative which is consistent with an increased contribution of random coil motifs (1). This suggests that the samples after incubation possess a higher content of random coils and virtually none of β-sheet. This depletion in β-sheet content shows that denaturation had occurred in a large extent.

At 70°C it appeared to occur irreversible denaturation of the Fc fragment of IgG while at 60°C the Fc fragment seems to suffer only minor alterations. Based on the fact that the percentage of Retained Biological Activity is determined on the affinity of Protein A and Protein G to the Fc fragment of IgG, it is clear that if the percentage of retained biological activity is diminished, the Fc fragment has suffered alterations since it no longer binds to Protein A or G.

Nonetheless, at 60°C there are mild changes in the secondary structure of IgG molecule, suggesting that other fragments of the IgG molecule may be affected upon incubation at this temperature. This situation may arise from IgG being a multi-domain protein which results on the fact of (at least) two existing domains denature at different temperatures.

3.1.2 pH induced denaturation

The pH induced denaturation has also been studied at two different ph, namely 2 and 3. After 50 hours of incubation at pH 3.02 at 4°C, it was only observed a residual loss of biological activity for the initial concentrations of IgG of 5 and 10 g/L. Upon 9 hours of incubation at pH 2.1, the percentage of retained biological activity was significantly lower when compared with pH ≈ 3, except for the initial concentration of 15 g/L where all IgG remained biologically active. The loss of activity was especially more pronounced for the concentrations of 5 and 10 g/L of IgG.

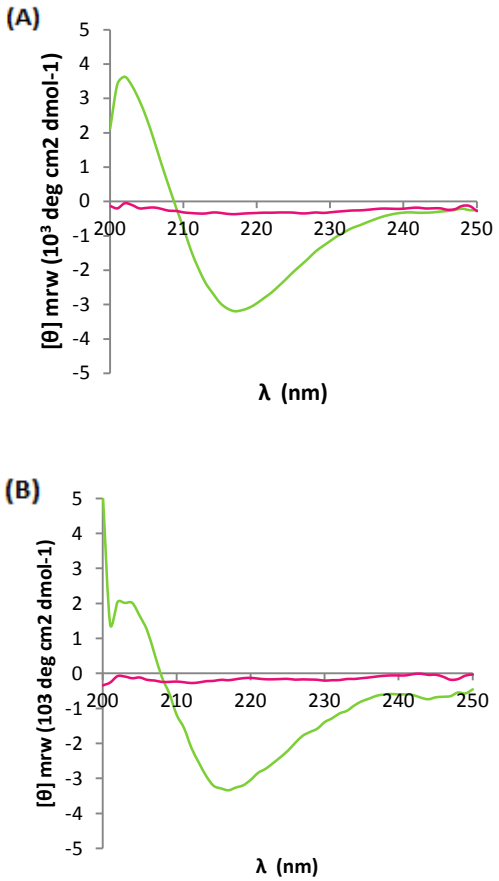

Figure 3 - Far-UV CD spectra of IgG in PBS (pH 7.4) before (—) and after 8 hours (—) of incubation at 70°C for the initial concentration of (A) 1 g/L and (B) 15 g/L.

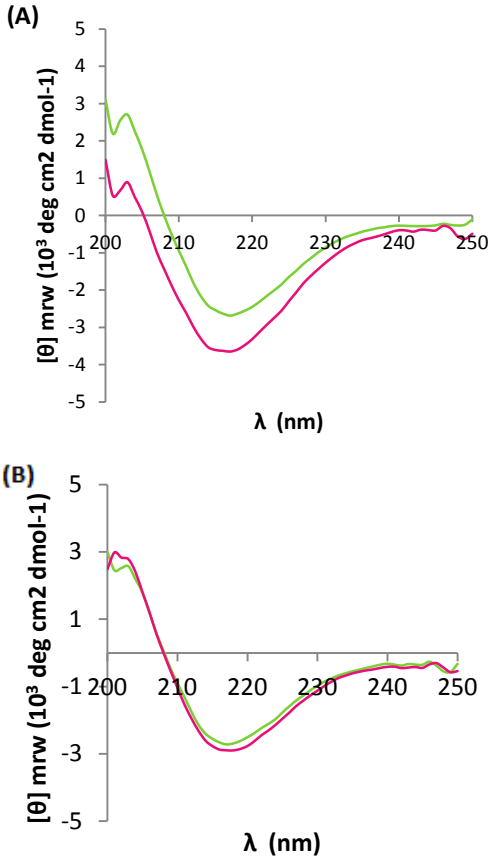

Figure 5 - Far-UV CD spectra of IgG in PBS before (—) and after 9 hours (—) of incubation at pH 2.1 for the initial concentration of 1 g/L (A) and 15 g/L (B).

The CD spectra were evaluated in order to determine whether changes in the secondary structure had occurred. Only the CD spectra in which the secondary structure suffered alterations are shown.

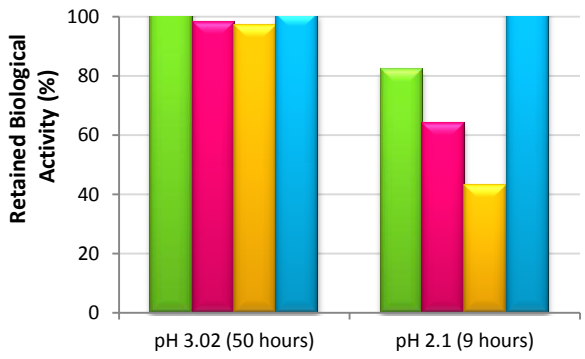

Figure 4 – Retained Biological Activity (%) after 50 and 9 hours of incubation of IgG solutions in PBS at pH 3.02 and pH 2.1, respectively, with the initial concentrations of 1 g/L (■), 5 g/L (■), 10 g/L (■) and 15 g/L (■).

After 50 hours of incubation in PBS at pH 3.02, the curves for the samples before and after incubation are for all purposes coincident, indicating that the secondary structure of IgG remains intact. Upon 9 hours of incubation at pH 2.1, for 15 g/L, the curves of the samples before and after incubation are superimposed, indicating that the secondary structure of the molecule didn't suffer alterations. The spectrum corresponding to the IgG solution with the initial concentration of 1 g/L (Figure 5) shows a curve after incubation with the same shape but lower values of ellipticity compared to the curve of intact IgG. This suggests that a change in the secondary structure has occurred but not in major extension. It has been reported the formation of a new, well-defined IgG structure upon exposure at low pH values (pH < 3). The protonation of amino acid side chains is supposedly causes the reorganization of the native state into a so called A-state, characterized by a high degree of secondary structure, increased accessibility of hydrophobic clusters, increased fluorescence intensity

and a native-like hydrophobic surrounding of the aromatic rings and described to be relatively compact [(17) , (18), (43)].

3.1.3 Mechanical Shear Stress induced degradation

Shear forces encountered by Taylor vortices can partition proteins to the air-water interface which encourages partial unfolding on exposure to the more hydrophobic air phase (3). The extent of the forces imposed on the IgG molecules during the assay are presented in Table 1.

Table 1 -Taylor number, shear rate (s^{-1}) and shear stress (Pa) imposed on the IgG molecules during the mechanical shear stress assay for the different angular speeds of the inner cylinder tested.

ω (rpm)	1000	2000	3000
Ta	1664	3327	4991
γ_m (s^{-1})	$9.40x10^3$	$2.66x10^4$	$4.88x10^4$
τ_m (Pa)	9	27	49

In all the assays conducted, the CD spectra were consistent with the behavior of a typical immunoglobulin, being the curves equivalent to high β-sheet content. The curves were similar in shape and ellipticity values suggesting that the IgG's secondary structure is maintained throughout the time spent in the shear field.

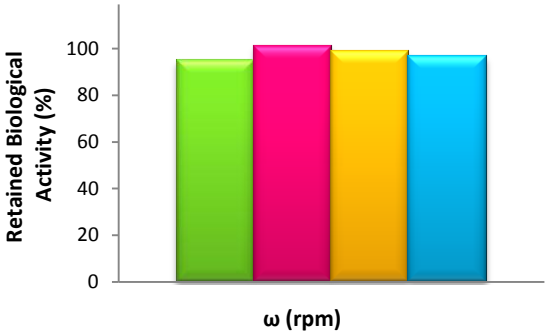

Figure 6 – Retained Biological Activity (%) after a period of circulation in the shear field of 30 minutes of IgG (1 g/l) at 1000 rpm (■), 2000 rpm (■), 3000 rpm (■)and IgG (15 g/l) at 3000 rpm (■).

The retained biological activity was of or close to 100% for all the situations (Figure 6), supporting the data obtained from CD spectroscopy. These combined data are strong indicators that the IgG molecule is resistant against mechanical sheer stress induced degradation, being able to sustain shear stresses up to 49 Pa.

3.2 Stability assessment of IgG with additives

To evaluate which of the selected additives is the best stabilizer, IgG solutions with each individual additive were incubated in the denaturation inducing conditions previously studied (70°C and pH ≈ 2).

3.2.1 Thermal induced denaturation

After 8 hours of incubation it was still observed a quite extent denaturation induced by heat, except for the initial IgG concentration of 1 g/L incubated with maltose in which all the IgG molecules remained biologically active. This means that maltose is able to fully stabilize IgG at this concentration against heat induced denaturation. Generally, all the values of retained biological activity obtained were higher in the presence of these stabilizers when compared with IgG in PBS. Figure 7 reveals that maltose is the more suitable stabilizer against thermal induced denaturation of IgG since it achieved the highest values of retained biological activity for most of the initial concentrations. It is also observable that the higher the initial concentration of IgG, the higher was the extent of the denaturation. For all the solutions under study, the formation of IgG precipitates after the 8 hours of incubation at 70°C were observed, as had already happened upon heat treatment of IgG in PBS.

Since the concentration of L-histidine was quite lower than the concentration of the other additives - 2% (w/v) compared to 10% (w/v) - it was evaluated whether maltose at 2% (w/v) would provide higher stabilization against thermal induced denaturation than L-histidine. Maltose still revealed a better behavior as a stabilizer of IgG against heat denaturation but the values of the retained biological activity for were significantly lower than for a concentration of 10% (w/v), which denotes the importance of the stabilizer concentration in the resistance provided against denaturation.

The CD spectra (Figure 8A-B) shows that, for both 1 and 15 g/L, the curve observed for the sample before incubation is consistent with a high β-sheet content. For 1 g/L, in spite of the ellipticity values between 200 and 220 nm of the sample after incubation being much lower and the minimum at 217 nm being a little broader when compared with the sample before incubation, the curve presents a similar shape to the initial one, indicating that some content of β-sheets is maintained. Nevertheless, at approximately 206.5 nm, where a typical β-sheet shows an intensity of zero, the curve presents a negative intensity at that wavelength, thus revealing the existence of random coil and maybe α-helix motifs (which exhibit negative contributions at that wavelength). For 15 g/L, the CD spectra show a slight improvement on the conservation of the secondary structure upon comparison with the CD spectra for IgG without any additive, since

the curve is somewhat similar in shape with the curve of a typical β-sheet motif. Even so, the distinction between the curves of the initial and final samples is fairly pronounced suggesting that changes in the secondary structure have definitely occurred.

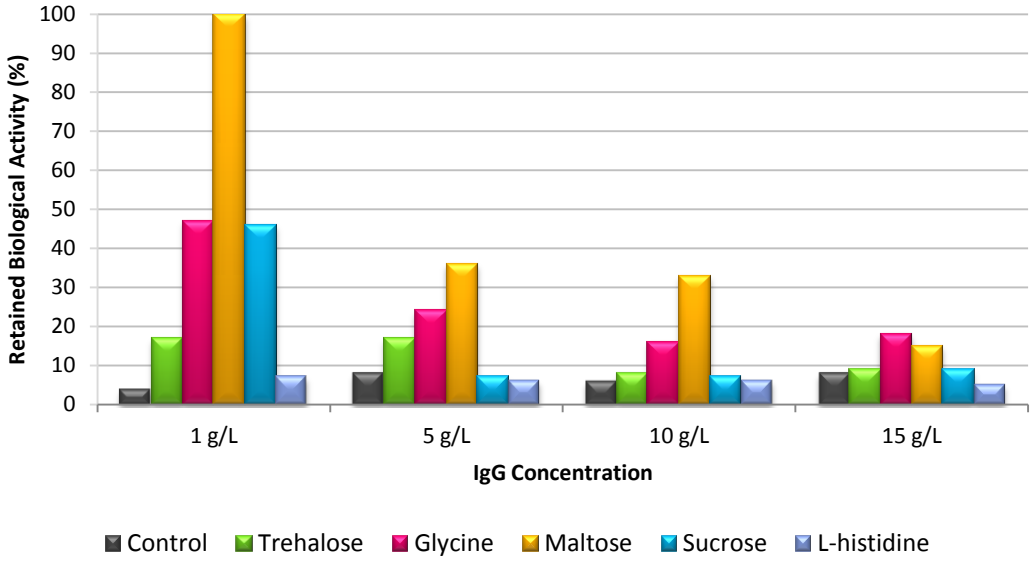

Figure 7 - Retained Biological Activity (%) after incubation at 70°C for a period of 8h of IgG solutions with the initial concentrations of 1, 5, 10 and 15 g/L. The buffers used consisted in PBS with 10% (w/v) of additive except for L-histidine in which the concentration of additive in the buffer was of 2% (w/v).

3.2.2 pH induced denaturation

Upon incubation at pH ≈ 2 in the presence of the several additives, it was observed some variability in the stabilization provided by the additives. Depending on the initial concentration of IgG in solution, the retained biological activity was largely different for the same additive. It can be seen in Figure 9 that maltose is also the best stabilizer against pH induced denaturation since it allows achieving a percentage of molecules biologically active after incubation equal or very close to 100% and it is, furthermore, the additive that showed less variability with the initial concentration of IgG in solution. The CD spectra exhibit curves identical to those of a typical immunoglobulin with high β-sheet content, with the curves of the samples before and after incubation virtually equal, suggesting that the secondary structure remains intact.

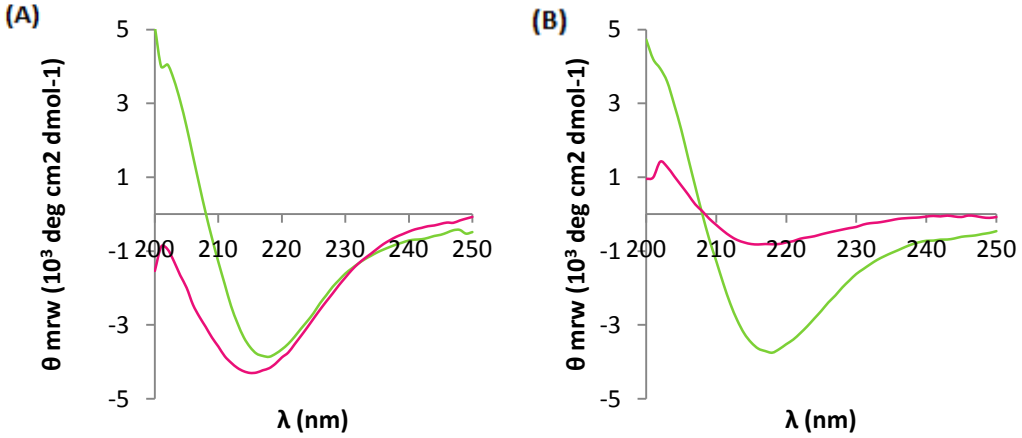

Figure 8 - Far-UV CD spectra of IgG in PBS with 10% (w/v) of maltose (pH ≈ 7) before (—) and after 8 hours (—) of incubation at 70°C for the initial concentration of (A) 1 g/L and (B) 15 g/L.

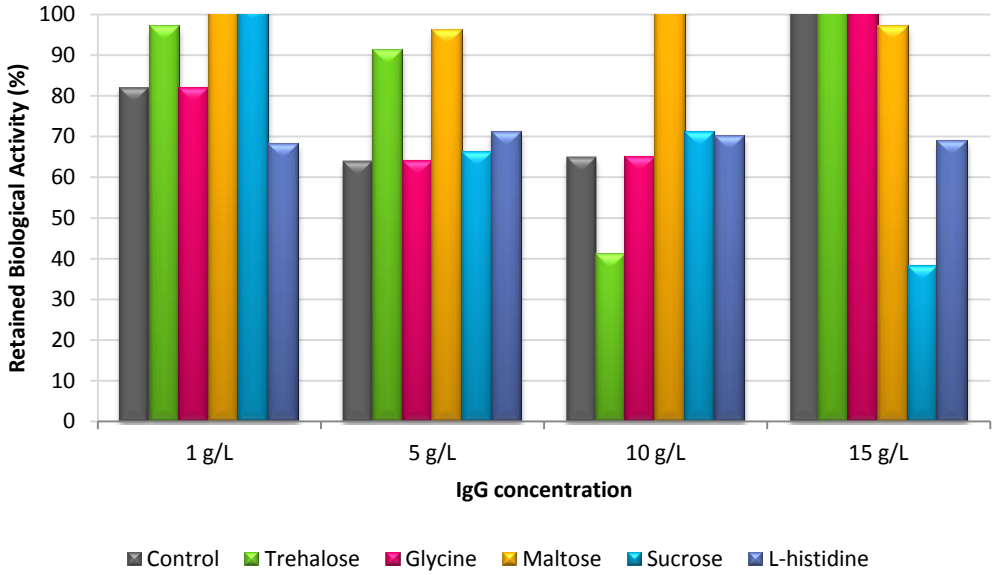

Figure 9 - Retained Biological Activity (%) after incubation of IgG solutions with the initial concentrations of 1, 5, 10 and 15 g/L at pH ≈ 2 for a period of 9h. The buffers used consisted in PBS with 10% (w/v) of additive except for L-histidine in which the concentration of additive in the buffer was of 2% (w/v).

Considering the results obtained both for the thermal and pH induced denaturation it is fair to conclude that maltose is the most suitable additive for IgG stabilization from amongst the additives studied. It has been recognized that sugars increase the transition temperatures of some proteins in aqueous solution, being this effect ascribed to the induction of a decrease in H-bond rupturing potency. Another source of stabilization is attributed to the preferential interaction of the protein with solvent components in the presence of high concentration of additives (11).

3.3 Aggregation Studies

Aggregation properties of IgG solutions are dependent on the conformational stability and structural properties of individual IgG molecules (19). In a large multi-domain protein like IgG, relatively gentle conditions can be sufficient to initiate aggregation. Partially unfolded states are much more susceptible to aggregation than the native or completely unfolded state, due to the exposure of contiguous hydrophobic regions that are hidden in the native state or absent in the denatured state (3).

The data presented for the pH assay correspond to the mode of the curves of the size distribution by volume. On the other hand, the size presented in the temperature assay corresponds to the average size measured. Since the presence of the larger precipitates may compromise the results by masking the size of smaller aggregates in solution, the most adequate approach would be to consider the contribution of all the different identities in solution.

3.3.1 pH effect on aggregation

When the pH is shifted away from the isoelectric point (in which all the molecules are electrically neutral), the molecules acquire charges – positive or negative, depending whether the pH is lower or higher than the isoelectric point. At high pH (pH ≈ 11), the IgG molecules are negatively charged since the pH is higher than the isoelectric point of IgG (pI ≈ 9). At low pH (pH ≈ 2), a similar situation occurs but, this turn, with positively charged molecules given that the pH is lower than IgG's isoelectric point. It is common knowledge that molecules with the same charge tend to repel each other, so it would be expected that with the increase of the molecule charge (either positive or negative) the tendency for aggregation would decrease. It is also expectable that the higher the initial concentration of IgG in solution, the lower the tendency for aggregation, since there are a higher number of molecules present in solution that may become charged causing more repulsion as result. It has been reported indeed that aggregation of polyclonal IgG decreases at lower pH, when distancing from the isoelectric range, while the tendency for denaturation increases (19).

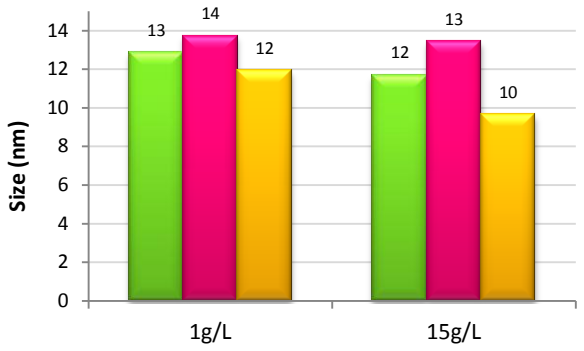

Figure 10 - Size (nm) of IgG in PBS at pH 2.1 before incubation (■) and after 9 hours of incubation in PBS at pH 2.0 (■) and PBS with 10% (w/v) maltose at pH 2.06 (■).

The tendency expected was not observed since, for both initial concentrations, the measured size after 9 hours of incubation was higher than for the sample prior to incubation (Figure 10). A size reduction would also be expected due to the formation of the previously mentioned A-state at low pH values which consists in a fairly compact structure. This may be due to the fact that these measurements were performed at a salt concentration of 150 mM NaCl, and thus charge shielding may have occurred. Nevertheless, in the presence of maltose, the particle size was somehow reduced. Regarding the samples incubated at pH ≈ 11 (Figure 11), a reduction in size was observed both in the presence and absence of maltose when compared to IgG at pH ≈ 11 at the beginning of the incubation. This goes along with the theoretical previsions. It is further observable that the size determined in the presence of maltose in solution was smaller than in its absence, especially at 15 g/L.

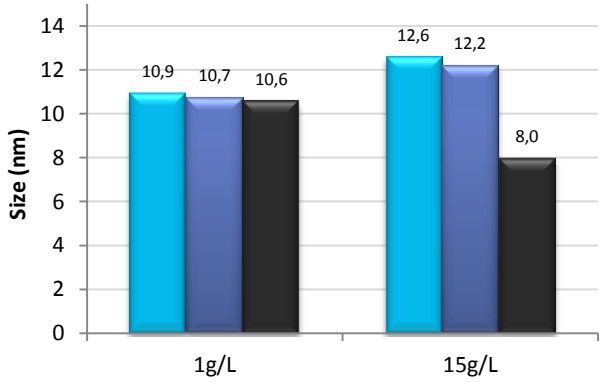

Figure 11 - Size (nm) of IgG in PBS at pH 11.3 before incubation (■) and after 9 hours of incubation in PBS at pH 11.3 (■) and PBS with 10% (w/v) maltose at pH 11.45 (■).

Maltose has the ability to inhibit the aggregation process both at low and high pH values in further extension than the pH effect alone (repulsion between molecules caused by the increase of the number of molecules with the same charge).

3.3.2 Temperature effect on aggregation

The reported size of native IgG is ≈ 10 nm (14) which is lower than the sizes determined experimentally (Figure 12). This difference may be ascribed as the result of long term storage at low temperatures of the commercial sample used, which is commonly accepted to happen (19).

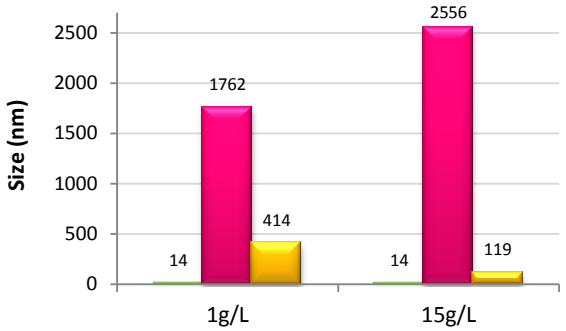

Figure 12 – Size (nm) of native IgG (■) and IgG after 8 hours of incubation at 70 °C in PBS (■) and PBS with 10% (w/v) maltose (■) – pH ≈ 7.

For both 1 and 15 g/L, the average size determined after incubation is much higher than the size of native IgG, which would be expected since the mechanism of heat denaturation comprises an aggregation step that leads to IgG precipitation. In the presence of maltose, the average size determined was significantly lower than in its absence which is in agreement with the results previously obtained: if aggregation is prevented to a certain extent, the heat denaturation is expected to be gentler. However, for 1 g/L the average size determined is higher than for 15 g/L which contrasts with the results obtained previously. Given that maltose provides higher stabilization for solutions with the initial concentration of 1 g/L against heat induce denaturation than for solution with a concentration of 15 g/L, the aggregation should be higher for the latter.

The variations of the particle size with time were plotted in terms of the fold increase in size (ratio between the particle size at a given time and the initial particle size) to express the aggregation rate (Figure 13A-B).

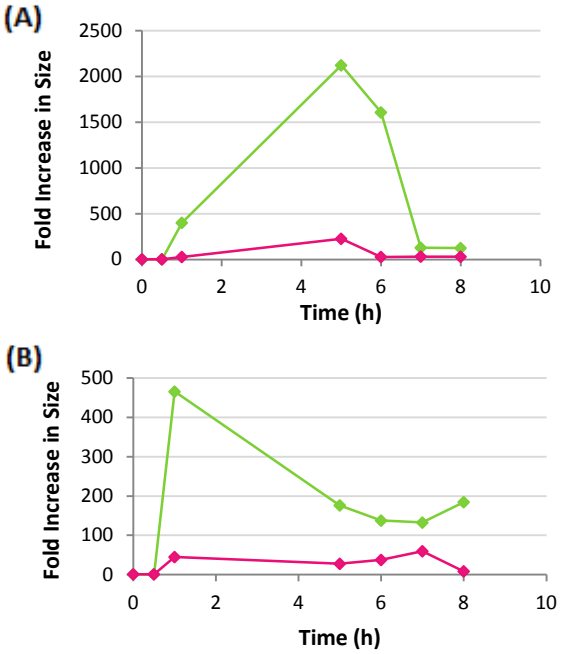

Figure 13 – Variations in size of IgG in solution over time of incubation at 70°C in hours for the initial concentration of IgG in solution of (A) 1 g/L and (B) 15 g/L, both in absence (—) and presence (—)of maltose. The Fold Increase in Size corresponds to the ratio between the size at a given time and the initial particle size.

It has been suggested in previous studies that at higher heating rate and/or incubation around or above the denaturation temperature, the unfolding occurs at a higher speed, leading to a high concentration of (partially) unfolded IgG molecules. The rate of aggregation is correspondingly faster, and it could well be that at such high aggregation rates IgG molecules are incorporated in the aggregate before they have had sufficient time for complete unfolding (16). In this experiment the incubation took place at a temperature near the denaturation temperature (T≈71°C for the Fc fragment (4)) and so the rate of aggregation would be expected to be high.

Even though the shape of the curves is significantly different, it is possible to verify the expected high rate of aggregation upon incubation in PBS alone for the two initial concentrations tested. In the presence of maltose, the rate of aggregation was significantly lower than in its absence, which correlates perfectly with the already done observation of maltose being able to stabilize IgG facing heat induced denaturation.

It is also important to refer that the decreasing aggregation tendency observed for the final period of incubation may be related to native domains initially present in solution being locked in the formed aggregates.

Maltose proved to give, once again, a positive contribution to IgG stabilization, being generally able to reduce the tendency for aggregation.

The addition of sugars to protein solutions is a popular approach to stabilize the protein and thereby reduce access to partially unfolded states that favor aggregation by hydrophobic contacts. Sugars are preferentially excluded from the surface of the protein hence favoring a compact state (3). Protein structures that exist in such a confined volume tend toward their state of minimum hydrodynamic volume, being the protein structure stabilized by a confined environment (17).

4. CONCLUSION

It was observed that IgG is stable upon pH induced denaturation at a pH of approximately 3 but, for lower pH values (pH ≈ 2) irreversible denaturation occurs in a certain extent. Regarding the thermal induced denaturation, IgG was found to be relatively stable at 60°C since that most molecules in solution maintain their biological activity. However, some gentle alterations in the secondary structure occur upon incubation at this temperature. When the temperature is increased to 70°C extensive irreversible denaturation occurs. Large IgG precipitates are also formed after heat treatment at 70°C, being this aggregation step the one that confers irreversibility to the heat denaturation process. Upon imposed mechanical shear forces, IgG was capable of maintaining its structure and remaining biologically active, being able to sustain shear forces up to 49 Pa. Finally, it was observed a tendency for aggregation upon incubation at 70°C at an elevated aggregation rate. It was observed a lower tendency for aggregation at elevated pH (pH ≈ 11), while for a low pH (pH ≈ 2) the particle size increased contrasting with the theoretical prevision of a lower tendency for aggregation at this pH range.

Maltose at a concentration of 10% (w/v) was found to be the best IgG stabilizer from amongst the additives studied. It accomplishes a full stabilization at low pH (approximately 2). It also allows a complete stabilization of IgG in a concentration of 1 g/L against heat denaturation at 70°C in terms of the ratio of IgG molecules that remain biologically active. However, the secondary structure showed some alterations after incubation which may only guarantee that the Fc fragment is stabilized in this situation. For the remaining initial concentrations it was still higher than in the absence of maltose. In general, maltose was also able to

reduce the tendency for aggregation both facing thermal and pH denaturation inducing conditions.

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