Stability of Chicken IgY Antibodies Freeze-Dried in the Presence of Lactose, Sucrose and Threahose

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Freeze-drying is used as a method to stabilize proteins. The process itself may however cause protein unfolding and denaturation, but the risk is reduced if a stabilizing agent is added prior to freeze-drying. Here chicken antibody (IgY) preparations were freeze-dried in the presence or absence of lactose, sucrose and threahose as stabilizing agent at 0.3, 0.06 and 0.012 M. The activity of freeze-dried IgY batches in the absence of stabilizing agent was similar before and after freeze-drying, but decreased slowly during eight weeks at -1°C. Addition of disaccharide resulted in a preserved activity after eight weeks in -1°C, compared to samples with no sugar added. The results were similar irrespective of sugar type and concentration (0.012–0.3 M). This shows that IgY freeze-dried in the presence of disaccharide is very stable and a method to reduce the cost and simplify transport, storage and use of avian antibodies.

Key words: ELISA, freeze-drying, IgY, lactose, sucrose, threahose


Introduction

Freeze-drying is a method to preserve proteins that are unstable in aqueous solution and the technique has frequently been used for pharmaceutical applications. Frozen samples are dried through removal of water by sublimation and desorption. Water is involved in several degradation processes (Manning et al., 1989; Cleland et al., 1993) that can be circumvented by water removal, but the freeze-drying process can itself lead to protein denaturation and protein loss (Chang and Fischer, 1995; Prestrelski et al., 1995; Allison et al., 1999). In addition, alteration of the protein structure can cause aggregation and denaturation during reconstitution (Hsu et al., 1995; Prestrelski et al., 1995; Zhang et al., 1995, 1996). Therefore it is common with addition of stabilizing agents such as polyols, sugars, amino acids, and surfactants prior to the freeze-drying process. Disaccharides are the only additives shown to protect unfolding during dehydration (Arakawa et al., 2001). The protein is protected by the sugar binding to hydrogen in substitution of water (Allison et al., 1999). Sugars also act protective already during the freezing process by increasing the free energy of unfolding leading to thermodynamic protection (Timasheff, 1998). In addition, sugars produce a glassy matrix protecting proteins during freeze-drying and subsequent storage through prevention of protein mobility and thereby also unfolding (Prestrelski et al., 1995; Duddu and Dal Monte, 1997). Long-term stability requires a native secondary structure in the dried state.

Chicken IgY is the functional equivalent to mammalian IgG, and IgY passes from the hen to the embryo via the egg yolk and therefore the egg yolk has a high concentration of chicken IgY. The “Y” in IgY comes from “yolk” and is the main antibody in the egg yolk. There is an increasing use of chicken IgY antibodies for diagnostic and thea-
tic purposes. The production of antibodies against conserved mammalian antigens, for diagnostic purposes, is in general more successful in chickens than in mammals due to the phylogenetical difference between birds and mammals. Chicken antibodies can also be used to reduce interference problems encountered due to anti-mammalian IgG antibodies present in many patients (Larsson et al., 1991, Larsson and Mellstedt, 1992). The most well known of these anti-mammalian IgG antibodies is the rheumatoid factor (RF) that react with the Fc portion of mammalian IgG. RF is usually found in serum samples from patient with rheumatoid arthritis, but can also be found in patients with other diseases and even in 3-5% of healthy individuals. In sensitive immunological assays up to 40% of the population may have interfering antibodies (Carlander et al., 1999). One other well-known antibody that can cause the same problems is the human anti-mouse IgG antibody (HAMA). An increasing number of patients are in vivo treated with mouse monoclonal antibodies and this often provokes an antibody response in the patient resulting in HAMA production. Thus, IgY offers definite advantage to the traditional antibodies in diagnostic assays.

Chicken antibodies have also been used for therapeu tic purposes. IgY directed against Pseudomonas aeruginosa has been used as peroral treatment in cystic fibrosis patients, who are predisposed for this bacterium (Carlander et al., 2003). Today IgY preparations are administered to patients as frozen batches to be thawed on the day of use. This is inconvenient for the patient and it is difficult to maintain the preparations frozen from production until use.

In this study we have investigated the possibility to freeze-dry IgY preparations as a way to simplify storage and transport requirements of antibodies for diagnostic and therapeutic applications. To reduce loss of antibody activity we tested the influence of the disaccharides sucrose, threalose and lactose as stabilizing agents, during freeze-drying, at concentrations of 0.012, 0.06 and 0.3 M. Since IgY is to be used as oral therapeutic, sugar is a good non-harmful alternative. Sucrose and threalose have been shown several times to have stabilizing effects on other proteins both during and after freeze-drying (Draber et al., 1995; Nielsen, 1995; Cleland et al., 2001; Garzon-Rodriguez et al., 2004). The effect of lactose as stabilizing agent is not studied to the same extent, but there are examples (Millqvist-Fureby et al., 1999; Omidfar et al., 2002). This is the first study on freeze-drying of IgY.

Materials and Methods

**Freeze-Drying**

Peroral IgY batches containing approximately 2 mg protein/mL with or without stabilizing agent were aliquoted into 1 mL fractions and frozen at −20°C. Three sugars; sucrose (BDH, Poole, England), threalose (D-(+)-threalose dehydrate, Sigma-Aldrich, Saint Louis, Missouri, USA) and lactose (α-D-lactose monohydrate, A.C.S reagent, Sigma-Aldrich) were used as stabilizing agents. The sugar concentrations used in the experiments were 0, 0.012, 0.06 and 0.3 M of each sugar. Frozen samples were then freeze-dried in a HETOTRAP CT-60 (Hetolab equipments A/S, Birkerod, Denmark) for approximately 24 hours (h). Thereafter, tubes were incubated at 37°C (forced stability test) or at room temperature (RT). After freeze-drying and incubation, at RT or in 37°C, samples were reconstituted in 1 mL MQ-H2O and stored at −20°C until further analysis was performed to reduce assay to assay variation. Samples were tested for IgY activity after 1, 2, 4 and 8 weeks storage at 37°C or 3 months storage at RT by ELISA. Samples frozen prior to freeze-drying served as controls.

**IgY-Activity ELISA**

96-well microtitre plates (F96 Polysorp, Nunc, Roskilde, Denmark) were coated with formaldehyde killed P. aeruginosa bacteria diluted 1 : 1000 in 0.1 M NaHCO3, pH 9.5 for 2 h at RT or over night at 4°C. Thereafter the wells were washed with 0.02 M NaH2PO4, 0.15 M NaCl, pH 7.2 (PBS) containing 0.05% Tween 20 (PBS-T) three times and blocked with 125 μL/well of 3 mg bovine serum albumin/mL in 0.1 M NaHCO3, pH 9.5 for 1 h on an orbital shaker at RT or over night at 4°C. The 100 μL of each sample diluted in PBS-T were added in duplicates after washing as previously described, and plates were incubated for 1h at RT. The plates were washed again as above and incubated for 1 h at RT with 100 μL horseradish-peroxidase- (HRP) Rabbit Anti- Chicken/Turkey IgG (IgY) (H+L) (Zymed, San Francisco, CA, USA) diluted 1 : 2000 in PBS-T. Bound secondary antibodies were detected with 100 μL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Zymed). The reaction was stopped after
ten minutes with 50 μL 1.8 M H₂SO₄. The absorbance was read at 450 nanometers in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). An ΔA₄₅₀ > 0.05 above background was considered significant throughout the experiment. The ELISA has a coefficient of variation of 7%. Differences below the coefficient of variation are considered within the margin of error of the ELISA.

Results

Activity of IgY after Freeze-Drying

The IgY activity of freeze-dried samples with or without sugar were tested after 1, 2, 4 and 8 weeks at 37°C or 3 months at RT by ELISA and compared to activity before (control) and after freeze-drying. Each time point was analyzed by duplicate samples and each sample were tested as duplicates in the ELISA plates. The activity was usually unchanged or slightly lower after freeze-drying than before. In most cases the reduction was within the margin of error of the ELISA. Sucrose at a concentration of 0.3 M and threalose at a concentration of 0.3 and 0.06 M showed slightly higher activity before freeze-drying. The samples containing 0.3 M sucrose or threalose were however difficult to reconstitute which might have resulted in less activity.

Activity of Freeze-Dried IgY Over Time

Without addition of a stabilizing agent the IgY activity decreased slowly over time compared to the control (Fig. 1) and after eight weeks in 37°C the activity was just below 80% of the control. Three different antibody batches were freeze-dried and they showed very similar results. When disaccharide was added the IgY activity was stabilized (Fig. 2). The activity of samples with sucrose was close to the freeze-dried and immediately frozen samples over time, except for the 0.3 M samples that retained 85% activity relative to control after eight weeks. For threalose there was a trend for increased activity after eight weeks when 0.3 and 0.06 M was added. Lactose stabilized the IgY antibodies to a slightly lesser extent than sucrose and threalose, but still the activity was higher than without the stabiliz-

Fig. 1. Stability of freeze-dried IgY after forced stability test at 37°C or 3 months at RT. The IgY activity was measured by ELISA and is expressed relatively to the freeze-dried sample stored in −20°C. The results of three batches in duplicates are shown. The activity of each batch before freeze-drying is also shown (control).

Fig. 2. Stability of IgY freeze-dried in the presence of disaccharide. (a) Sucrose, (b) threalose and (c) lactose at 0.012, 0.06 and 0.3 M was added prior to freeze-drying. IgY activity was measured by ELISA after forced stability test (1, 2, 4 and 8 weeks at 37°C) and after 3 months at RT. Activity is expressed relative to activity immediately after freeze-drying. Samples frozen prior to freeze-drying served as controls.
ing agent and close to the control.

**Discussion**

Here we investigated the stability of chicken antibodies (IgY) after freeze-drying with or without stabilizing adjuvant. Forced stability test at 37°C during eight weeks showed that the activity was slowly reduced over time for the batches freeze-dried in the absence of sugar. The decrease in activity is still not so high that the IgY preparation should lose their function.

Three batches were tested without stabilizing agent and since the individual differences were limited, only one of them was freeze-dried with each concentration of disaccharide. The ability of the sugar to serve as a stabilizing agent can vary depending on the protein that is freeze dried. Here sucrose and threalose were almost equally good stabilizers while lactose was slightly less effective. IgY-preparations freeze-dried with lactose may also be less suited for oral administration to humans as reduced lactase activity is a frequently occurring condition that may result in intolerance to a lactose containing IgY preparation. Sucrose and threalose are both not harmful and have been shown to effectively stabilize different types of antibodies during freeze-drying and subsequent storage (Draber et al., 1995; Nielsen, 1995; Cleland et al., 2001).

The highest concentration tested, 0.3 M was difficult to reconstitute and since the effect was not clearly better than with lower concentration it is recommended to use lower sugar concentrations. Sucrose is cheaper and therefore the firsthand choice as a stabilizing agent. One can imply that an increased risk of caries would appear if IgY preparations should be gaggled after tooth brushing in the evening, but we found that even the lowest concentration, 0.012 M was effective as stabilizer and such a low concentration should be acceptable. It has been indicated that high doses of sucrose in intravenous immunoglobulin can cause renal injury (Winward and Brophy, 1995), but that should not be the case with low concentrations such as 0.012 M. In fact there was a tendency for better stabilization with 0.012 M than with 0.3 M sucrose after eight weeks at 37°C. One week storage at 37°C is comparable to one year at 4°C or eight weeks at room temperature, which means that the storage of IgY at 37°C for eight weeks corresponds to more than one year at room temperature (Baertschi, 2005). Therefore IgY freeze-dried with sugar in this study is very stable for more than a year at RT and for at least three months in the absence of sugar.

Murine IgG antibodies that were freeze-dried with sucrose, as one among other lyoprotectants, had a reduced antigen-binding of about 10% (Ressing et al., 1992). In our study the reduction was usually less and within the margin of error of the ELISA. This may be in agreement with previous studies reporting that IgY had a more stable molecular structure than mammalian IgG (Warr et al., 1995). Peroral anti- *Pseudomonas aeruginosa* IgY preparations are used therapeutically by a group of Swedish cystic fibrosis patients. Freeze-dried IgY would simplify the use since the present aqueous solution has to be frozen until time of use. Instead of thawing a bottle before use the patient could just reconstitute the freeze-dried powder with water. There are also studies showing a protective effect of IgY against other bacterial, fungal, viral and parasitic infections (Hatta et al., 1997; Sarker et al., 2001; Kobayashi et al., 2004; Suzuki et al., 2004). This points at a high potential for therapeutic use of IgY in many diseases, but that requires stability in room temperature for easier and cheaper use. IgY can also be used for diagnostic purposes. A freeze-dried antibody preparation may also increase the shelf life of such antibody preparations.

In summary our results shows that IgY preparations are stable for a long time when freeze-dried in the presence of disaccharide and that it was sufficient with the lowest concentration tested, 0.012 M. The possibility of freeze-drying would simplify transport, use and storage of IgY and also reduce the cost.

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**References**


