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Lyoprotection of aviscumine with low molecular weight dextrans

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Abstract

The aim of this research was to ascertain whether dextrans with low molecular weight will stabilize aviscumine. During freeze-drying increasing concentrations of dextran T1 (MW 1000) stabilized aviscumine. Eight percent of dextran resulted in a nearly 100% recovery of the activity and in addition a complete amorphous structure of the solid phase was obtained. By decreasing the molecular weight of the dextran from 75 to 1 kDa, the protein activity was increased by 20% in the lyophilisate. Combinations of dextran with either trehalose or mannitol showed no additional effects on stability. The improved stabilization of aviscumine using low molecular weight dextrans is explained by an increased interaction between the protein and the dextran molecules (like hydrogen bonds), whereas they are sterically hindered if larger dextran molecules are used. When the protein concentration was increased from 10 to 100 μg/ml (in formulas with 8% dextran T1), no influence on the protein activity could be found. With regard to the carbohydrate-binding activity of the protein, it was shown that the optimal content of residual water in the lyophilisate should be about 2%. Above and below this percentage a destabilization of the protein was observed. The often discussed failure of dextran as a stabilizing excipient in the freeze-drying of proteins seems to be a question of the selection of the correct molecular weight.

Keywords: Aviscumine; 223577-45-5; Lyophilization; Stability; Dextran; Molecular weight

1. Introduction

Aviscumine (prop. INN, CAS No.: 223577-45-5, scientific name: rViscumin) is a heterodimeric plant-derived protein from the European mistletoe *Viscum album* L. manufactured by recombinant DNA technologies. The protein combines specific carbohydrate-binding properties located in the B-chain (specific for α-2,6-sialolactosamine (Müthing et al., 2002) and terminal galactose) with ribosome inactivating properties in the A-chain (28S-rRNA-*N*-glycosidase) and belongs to the class II of ribosome inactivating proteins (Lentzen et al., 2001). Due to both activities aviscumine is cytotoxic to cells and is classified as an antineoplastic agent (Möckel et al., 1997; Eck et al., 1999). The protein is a new active substance which is under clinical investigation for cancer treatment.

The stability of the aqueous solution, however, was only suitable to realize the clinical trials in phase I. Due to the stability of aviscumine at higher pH values, the pH of the drug product solution was adjusted at 8.0 (Witthohn et al., 2002). Freeze-drying (lyophilization) is a commonly used technique in order to obtain acceptable shelf life for protein pharmaceuticals (Franks, 1998). The process, however, generates

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freezing (e.g. freeze concentration, formation of ice crystals, pH changes) and drying stresses, as well as during reconstitution, towards a stress-specific stabilization with a variety of excipients that has to be addressed (Carpenter et al., 1993; Webb et al., 2002). Polymers, like dextran, fail to stabilize proteins during freeze-drying although their high glass transition temperature makes them predestined as lyoprotectors. These excipients seem to maintain only an amorphous cake structure without developing interactions to the protein which are absolutely necessary for the protein recovery (Carpenter et al., 1993; Carpenter and Izutsu, 1999; Allison et al., 1999, 2000). Furthermore, it is known that the carbohydrates stabilizing capacity (e.g. dextran) decreases with increasing molecular weight (Tanaka et al., 1991). Therefore, the aim of this research was to study if dextrans with low molecular weight are able to stabilize aviscumine in freeze-dried formulas. In order to optimize protein recovery, different concentrations of dextran and protein were used. To conclude the stabilizing mechanism, the coherence between stabilization and molecular weight of different dextrans was investigated. Also, the stabilizing effect of combinations of dextran with trehalose or mannitol was assayed. The carbohydrate-binding activity of aviscumine, the crystallinity, the water content of the lyophilisates, and the stability at elevated temperatures served as appraisal factors.

2. Materials and methods

2.1. Materials

Aviscumine, precipitated in 2.67 M ammonium sulfate (storage at 2–8 °C), and anti-aviscumine antibodies were provided by VISCUM AG (Zwingenberg, Germany). Dextrans T1, T5, T10, T40, and T60 were purchased from AMERSHAM PHARMACIA BIOTECH AB (Uppsala, Sweden). Tris(hydroxymethyl)aminomethane, sodium chloride, potassium chloride, sodium-EDTA, and hydrochloric acid were purchased from Merck AG (Darmstadt, Germany). Polysorbate 80 was obtained from UNIQUEMA BV (Everberg, Belgium). BSA, streptavidin POD, OPD, asialofetuin (type I) from fetal calf serum, *N*-acetylneuraminic acid <0.5% (product no. A4781), and anti-mouse POD were obtained from SIGMA-ALDRICH CHEMIE GmbH (Steinheim, Germany). The water used for all solutions corresponds to the Ph.Eur 1997 monograph “water for injection”.

2.2. Preparation of bulk solution and freeze-drying

The different dextrans were dissolved in a solution of Tris (100 mM), sodium-EDTA (10 μg/ml), and polysorbate 80 (1 mg/ml) in water. The solution was adjusted to pH 8.0 with 1N HCl. Aviscumine was dissolved in this solution to a concentration of 10 μg/ml. A 0.5 ml of the bulk solution was filled in transparent glass vials (type I glass, Forma Vitrum AG, St. Gallen, Switzerland) and preclosed with a lyophilization stopper (WEST COMPANY, Aachen, Germany). The vials were placed in the freeze-dryer (GFT6, Klein Vakuumtechnik GmbH, Niederfischbach, Germany) at room temperature, and the temperature of the freeze-dryer shelves was decreased to 0 °C for 3 h followed by a cooling to −35 °C for 12 h. Samples (three vials) of each batch were removed in order to determine the loss in protein activity after freezing (freeze–thawing procedure). The primary drying was performed by raising the shelf temperature to −10 °C in 8 h, followed by increasing the temperature in 8 h to 10 °C (all at 80 kPa). The secondary drying started with a raising of the temperature to 30 °C within 1 h, followed by a drying for 6 h at 30 °C (all at 10 kPa). Finally, the freeze-dryer was flushed with sterile air and the vials were closed directly in the apparatus.

2.3. Stability at elevated temperatures

The stress stability test was carried out at 60 °C in a water bath for 8 h. Every 2 h three samples from each formula were drawn from the water bath and stored at 2–8 °C until analyzed with an ELLA.

2.4. Assays

2.4.1. Activity of aviscumine

2.4.1.1. Carbohydrate-binding activity (ELLA). The carbohydrate-binding activity of the protein was measured by a modified ELISA: Asialofetuin (glycoprotein with terminal galactose residues) was fixed to a micro titer plate; aviscumine was bound to the

glycoprotein and was detected with a monoclonal antibody. The orange-colored end product of the oxidation of *o*-phenylenediamine (catalyzed by horse radish peroxidase coupled to the secondary antibody) was used for the photometrical detection at 492 nm (Tecan Spectra Thermo, Tecan, Crailsheim, Germany). The relative activity was calculated by comparing the freshly prepared solution with the freeze–thawed solution and with the freeze-dried product after reconstitution with water, respectively. A mean value of four independent vials represented the given carbohydrate activity data. The statistical verification was done with ANOVA (Statistica, 5.0). The robustness of the assay was tested with the aqueous solution of aviscumine. The recovery of samples spiked with aviscumine to a total concentration range of 40–140 ng/ml was in the range 95–105%. The accuracy and precision determined with a concentration of 100 ng/ml with six independent solutions in six titer plates was 106.7 ng/ml with a relative S.D. of 7.1%. The maximal deviation from the mean was determined to be 93.2 and 113.6%.

2.4.1.2. Drug activity (ELISA). The drug activity was determined with an ELISA using two different monoclonal antibodies for capture (anti-B-chain aviscumine IgG antibody, mice) and detection (anti-A-chain aviscumine IgG antibody, mice). The asymmetric sandwich complex was detected photometrically according to the ELLA method as well as the number of measured samples, calculation of the values, and their statistical analysis. The robustness of the assay was tested with the aqueous solution of aviscumine. The recovery of samples spiked with aviscumine to a total concentration range of 30–100 ng/ml was in the range 100–112%. The accuracy and precision determined with a concentration of 40 ng/ml with six independent solutions in six titer plates was 41.9 ng/ml with a relative S.D. of 5.9%. The maximal deviation from the mean was determined to be 91.2 and 107.2%:

2.4.2. Water content of the lyophilisates

Karl Fisher titration (Metrohm, Herisau, Germany) was performed in a glove box (adjusted to a relative humidity of 20% with silicone gel). The sample (20–40 mg, equivalent to the content of one vial) was weighed in the glove box, transferred into the titrator (solvent: Apura®-Combisolvent, Merck AG, Darmstadt, Germany), stirred for 3 min, and titrated immediately with CombiTitrant® 1 (Merck AG, Darmstadt, Germany).

2.4.3. DSC

Glass transition temperature (T_g') was measured using differential scanning calorimetry DSC 7 with integrated cooling cell CCA7 (Perkin Elmer, Norwalk, USA) using liquid nitrogen as coolant. The temperature range of analysis was between −80 and 15 °C (heating rate: 5 K/min, cooling rate: 10 K/min). About 20 mg solution was weighed in a sealed pan and scanned for three cooling and heating cycles. The glass transition temperature was determined corresponding to the method described by Levine and Slade (1988) using the first deviation of the DSC scan.

2.4.4. X-ray diffractometry

The characterization of the lyophilisates was carried out by using a rotating anode X-ray diffractometer (Stoe & Cie GmbH, Darmstadt, Germany) with Cu Kα1 radiation (monochromator: germanium) generated at 8 kW. The powder samples were packed between Mylar® foils and rotated. The X-ray pattern was measured by a position-sensitive detector (PSD, Stoe & Cie GmbH, Darmstadt, Germany) in transmission. The estimation of crystallinity was achieved by comparison of the areas under the curves of crystalline and partially amorphous materials (Hops, 2000).

3. Results and discussion

The stabilizing effect of dextran T1 on aviscumine with respect to the carbohydrate-binding activity (ELLA) is shown in Fig. 1. During freezing (white bars) the activity of aviscumine was not influenced by dextran independently to its concentration. In contrast, the freeze-dried solid (filled bars) showed a tremendous degradation after reconstitution in the dextran-free preparation (50%) or at low dextran concentrations. It is obvious from this result that the buffer system plus polysorbate 80 alone showed sufficient cryoprotective efficiency, whereas dextran only had a lyoprotective effect. In the case of dextran T1, this effect was pronounced at concentrations above 4%. At this concentration there was no significant degradation of the protein between freezing and

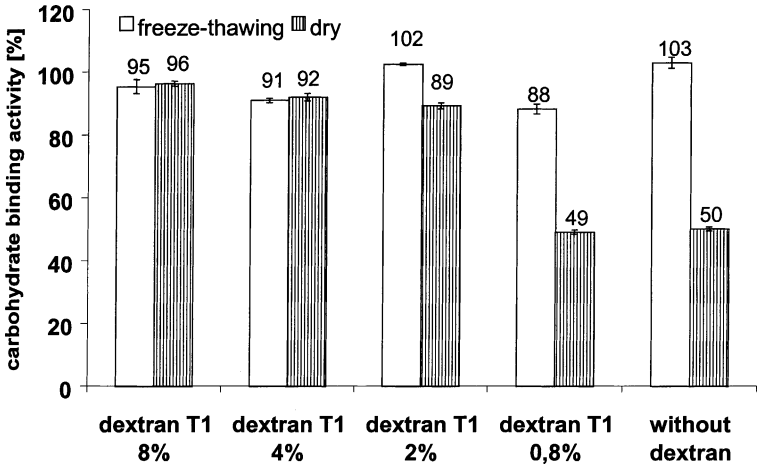

Fig. 1. Stabilization of aviscumine (10 μg/ml) during freeze-drying of aqueous formulations with dextran T1 (in 100 mM Tris buffer, pH 8.0, with 0.1% Tween® 80) ($n = 4$; S.D. = error bar).

drying steps of the lyophilization ($P \leq 0.2115$ for dextran 4% and $P \leq 0.4368$ for dextran 8%), whereas at a dextran concentration of 2% there was a significant degradation ($P \leq 0.0004$). So aviscumine is stabilized during freezing by the buffer system plus polysorbate 80 and during water evaporation in the drying step by the dextran. Dextran concentrations lower than 4% revealed no sufficient stabilization while the addition of 8% led to a product with a protein activity near 100%.

In Fig. 2 the X-ray powder diffractograms of the lyophilisates are shown. Fig. 2a represents the patterns of the dextran-free formulation showing a complete crystallization of the freeze-dried solid material. Without dextran as excipient the Tris buffer system crystallized completely giving unstable products. It is evident that the crystallization of the buffer components damages the secondary structure of the hydrated protein. In the case of Fig. 2b, the addition of 2% of dextran T1 did not sufficiently prevent this crystallization of the buffer components. Thus, an increase in the drug stability can be observed; however, the degradation still exists. Four percent of dextran T1 in the formulation led to an amorphous cake structure of the freeze-dried solid (Fig. 2c), showing a sufficient protein stability of the product.

The separate crystallization of a single component causes a phase separation of the excipients and the protein. As a consequence, the protein molecule loses its binding properties to the stabilizing excipient in the freezing step. But a stabilization in the dry state by water replacement can only occur in a consistent amorphous phase (Carpenter et al., 1993), which can be obtained in this case by a sufficiently high dextran concentration (above 6%). Furthermore, the amorphous status of a freeze-dried protein formulation prevents the surface denaturation which is observed at the surface site of crystals. Therefore, in amorphous cakes the protein stability is increased (Chang et al., 1996). Besides the water replacement theory, the concentration-dependent stabilization by low molecular weight dextran can also be explained with the "glass forming theory" by Levine and Slade. In the glassy matrix relaxing and rotating molecule transitions are kinetically immobilized and the protein is "diluted", so that denaturation (e.g. aggregation and agglomeration) is reduced by separation of the protein molecules (Levine and Slade, 1988). In the glassy state the protein activity may also be maintained by a reduced pH shift of the buffer or by an inhibited dissociation of the protein molecule during primary drying (Anchodoquy and Carpenter, 1996; Anchodoquy et al., 2001). Due to the basic pH optimum of aviscumine (Witthohn et al., 2002), and owing to the fact that a Tris buffer shifts towards a basic pH value during freezing (Gloger et al., 2003, in press), the stabilization effect of dextran on aviscumine may be caused by a reduced dissociation of the protein.

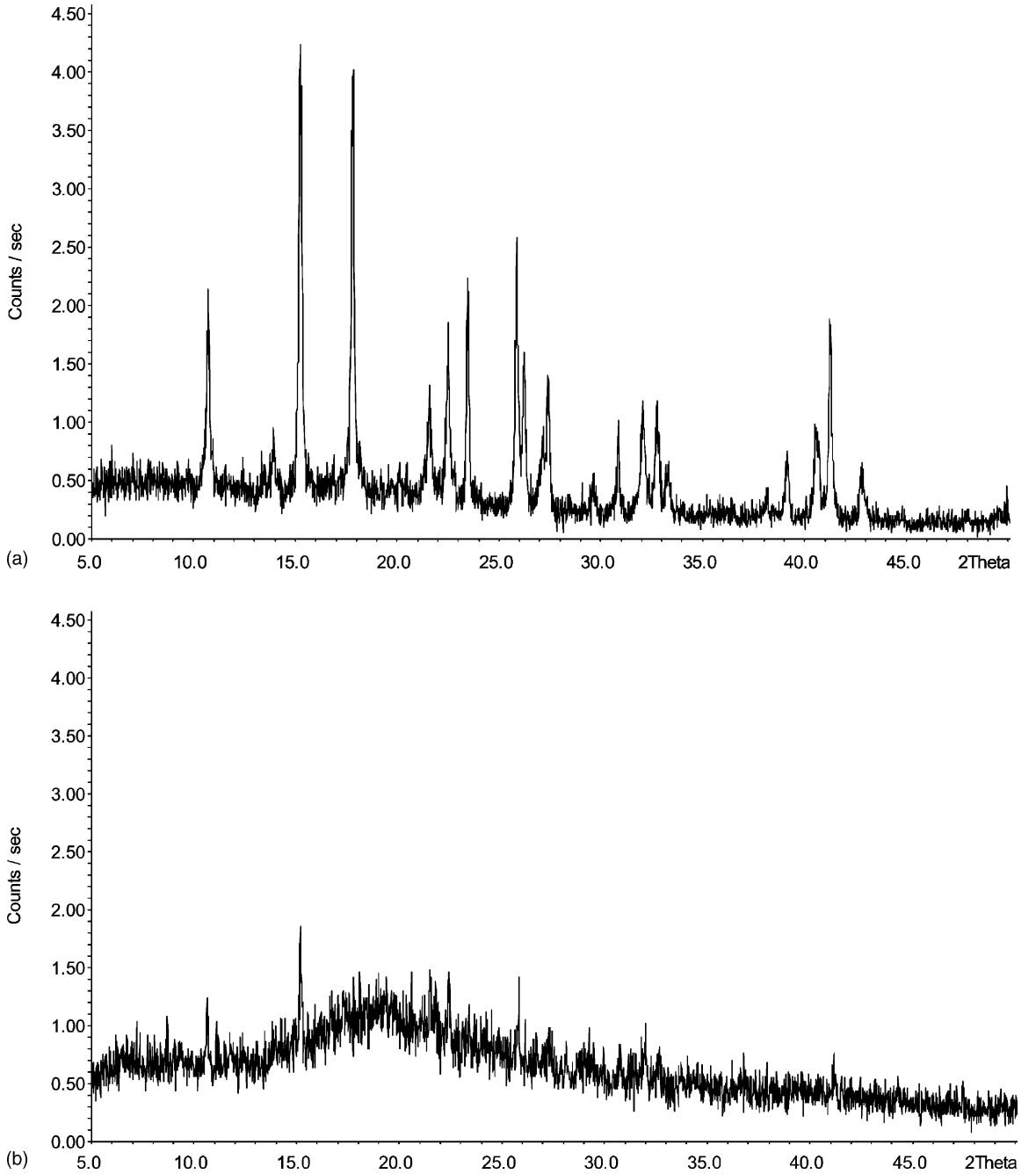

Fig. 2. X-ray powder diffractograms of aqueous formulations of aviscumine (10 μg/ml) in 100 mM Tris buffer, pH 8.0, 0.1% polysorbate 80 without dextran T1 (a), with 2% dextran T1 (b), and with 4% dextran T1 (c).

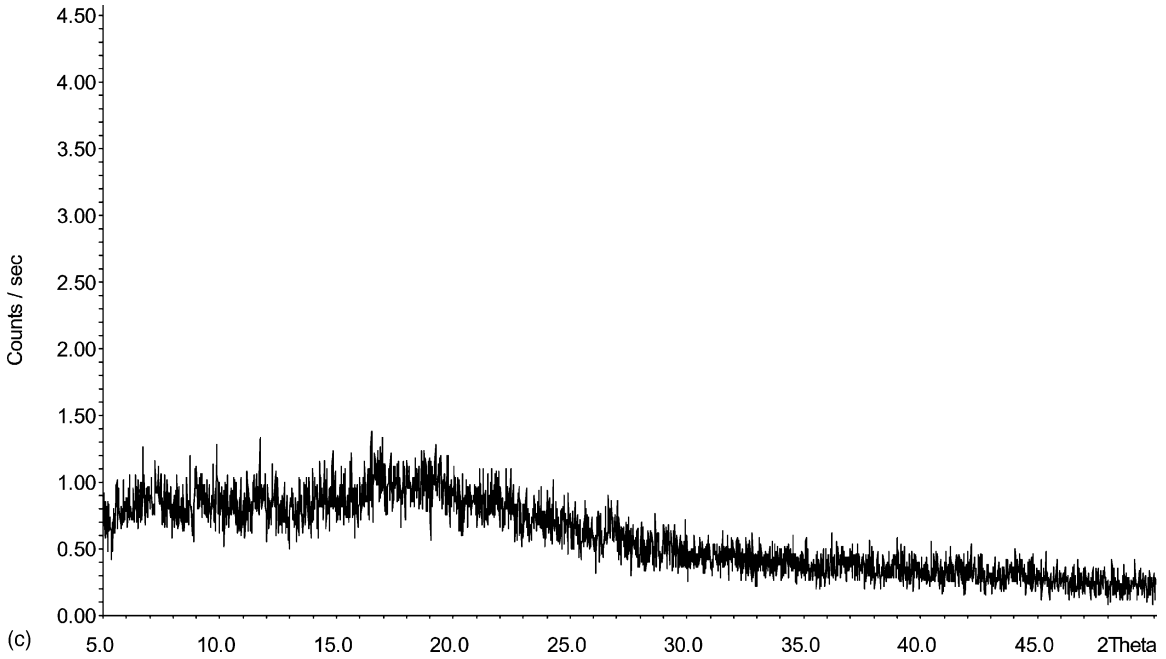

Fig. 2. (*Continued*).

In the literature there are, however, several publications which report the failure of dextran in protein stabilization (Carpenter et al., 1993; Carpenter and Izutsu, 1999; Allison et al., 1999, 2000). Following their results, this polymer only creates an amorphous cake structure which is a necessary but not sufficient condition to obtain full protein recovery. A combination of dextran with trehalose or sucrose could possibly close the "gap" between the protein and the excipient because these small carbohydrates interact in a better way with the proteins (Allison et al., 2000). In the case of aviscumine, these findings could not be repeated. Fig. 3 reveals that the addition of trehalose has no concentration-dependent effect on the

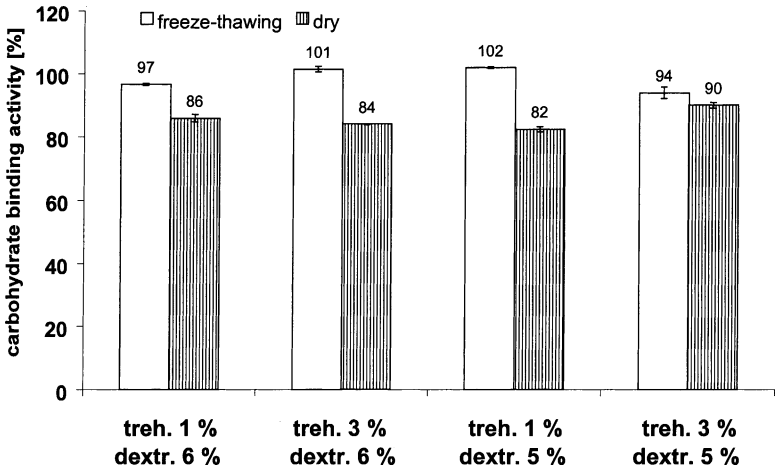

Fig. 3. Influence of trehalose on activity of aviscumine freeze-dried in 100 mM Tris buffer, pH 8.0, with 0.1% Tween® 80 and dextran T1 ($n = 4$; S.D. = error bar).

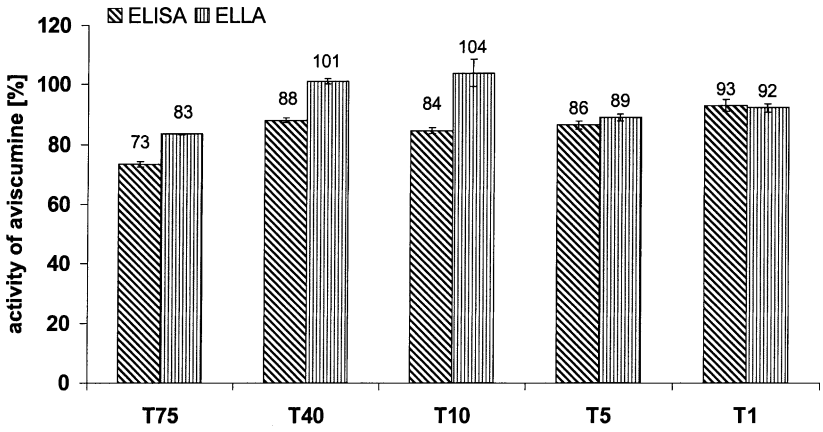

Fig. 4. Effect of molecular weight of dextran (4% of each dextran in 100 mM Tris buffer, pH 8.0, 0.1% Tween® 80) on drug activity (ELISA) and carbohydrate-binding activity (ELLA) of freeze-dried aviscumine ($n = 4$; S.D. = error bar).

carbohydrate-binding activity of dried aviscumine. The effect of trehalose on the residual moisture of the lyophilisate is discussed further on.

In order to explain the discrepancy between the results in this study and those from the literature, dextrans with different molecular weights were investigated (Fig. 4). When the molecular weight of the dextran was decreased from 75 to 10 kDa in formulas prepared in 100 mM Tris buffer, pH 8.0 (dextran concentration 4% (m/v)), the carbohydrate-binding activity of aviscumine (vertical filled bars represent the ELLA) increased to 21% ($P \leq 0.0237$) in the dried product. Using the small dextrans with 1 or 5 kDa molecular weight resulted in a carbohydrate-binding activity of only 90%. However, it has to be recognized that in a pharmacologically active aviscumine molecule the carbohydrate-binding activity as well as the protein-binding activity should remain intact (Endo et al., 1988; Eck et al., 1999). The first is investigated by using the reported ELLA test while the latter is analyzed by an ELISA test. Discrepancies between the results of both assays lead to a reduced pharmacological effect of the protein. Due to both activities only aviscumine, as a class II ribosome inactivating protein (Lentzen et al., 2001), can act in its specific cellular mechanism (Endo et al., 1988; Decastel et al., 1989). In spite of the lower recovery of the carbohydrate-binding activity (e.g. 92% for dextran T1 versus 104% for dextran T10 in Fig. 4 vertical filled bars), the low molecular weight dextrans caused a better stabilization of the protein-binding site (e.g. 93% for dextran T1 versus 84% for dextran T10 in Fig. 4 diagonal filled bars). Therefore, the samples prepared with the “small” dextrans (1 and 5 kDa) are more suitable than those with dextrans of higher molecular weights (10, 40, and 75 kDa).

It is known from the literature that the stabilizing capacity of carbohydrates, such as dextrans, decreases with increasing molecular weight (Tanaka et al., 1991). This is explained by a sterical hindrance of the binding (by hydrogen bonds) between drug molecule and excipient. High molecular weight dextrans show more intramolecular hydrogen bonds which lead to a loss in intermolecular bonding capacity. This results in a decreased protein stability (Tanaka et al., 1991). So the increased protein stability in preparations with low molecular weight dextrans can be very well explained.

In addition, the stabilizing capability of dextran T1 was investigated in preparations with increasing aviscumine content (Fig. 5). Although the cryoprotection of the protein significantly ($P \leq 0.0124$) increased with a higher concentration of aviscumine during freezing (the formulas with 10 or 100 μg/ml aviscumine were compared), the protein activity was not stabilized during drying. In contrast, the activity decreased to about 8% ($P \leq 0.0498$) with the increased protein concentration. Therefore, there was no further significant stabilization in the dried state due to a higher protein concentration in preparations with 8% dexran T1.

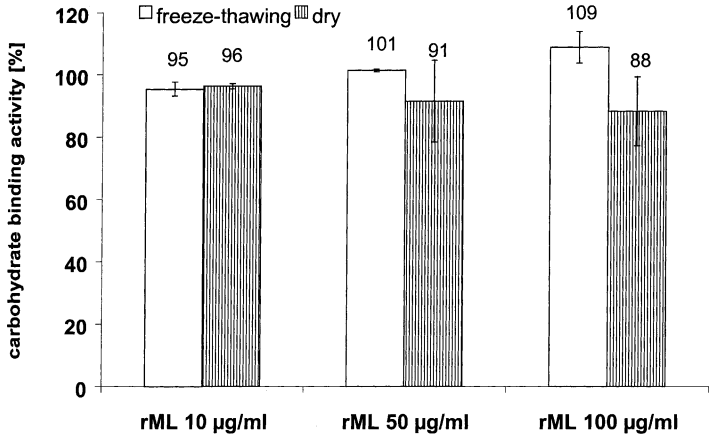

Fig. 5. Influence of protein concentration on activity of aviscumine freeze-dried in 100 mM Tris buffer, pH 8.0, with 0.1% Tween® 80 and 8% dextran T1 ($n = 4$; S.D. = error bar).

The water content of preparations with low and high molecular weight dextrans is listed in Table 1. Increasing the dextran concentration from 2 to 8% lowered the moisture content of the 3.4% dried product from 5.4 to 2.3% ($P \leq 0.0012$). High molecular weight dextrans lead to higher water contents of the lyophilisates. However, it is known that proteins need a certain amount of water to develop optimal stability. Overdrying would destroy proteins as would a higher water content (Jiang and Nail, 1998; Pikal et al., 1991). For aviscumine about 2% of residual water in the freeze-dried solid will lead to an optimal carbohydrate-binding activity. This could be reached with the preparation containing 8% dextran T1. The above discussed failure of dextran to stabilize proteins may be caused not only by the decrease in binding sites between drug molecule and excipient but may also be due to the high content in residual water in relation to the use of dextrans with higher molecular weight (e.g. 75,000 dextran, Table 1).

The increased effect on stabilization of proteins by mixing dextrans with trehalose as suggested by Allison et al. (2000) could not be repeated with avisumine (Fig. 3). One explanation of this result can be obtained from Table 2. Compared with the optimal residual water for aviscumine co-freeze-dried with 2% dextran, the freeze-dried product with 5% dextran and 3% trehalose is too dry (1.8%, $P \leq 0.0009$). The residual water content in the freeze-dried product prepared with trehalose is too low.

Besides trehalose, mannitol is also suggested in combination with dextran as excipient for protein lyophilization (Hora et al., 1992). During freeze–thawing 6% ($P \leq 0.0529$) of the activity was lost but during drying nearly 40% ($P \leq 0.0004$) of the activity was lost if mannitol was used as stabilizer (Fig. 6). The addition of dextran enhanced protein activity during drying by 13% (from 61 to 74%, $P \leq 0.00309$), but this result is less if compared with the preparation with dextran as single lyoprotector in Fig. 1. Powder diffractometry as well as DSC (data

Table 1
Water content of selected formulas with different dextran types ($n = 2$)

Dextran type	Dextran concentration [%]	Water content [%]	S.D.
T1	2	5.4	0.14
T1	4	3.5	0.16
T1	8	2.3	0.04
T75	4	4.4	0.35

Table 2
Water content of formulas with different dextran trehalose combinations ($n = 2$)

Dextran trehalose combination	Water content [%]	S.D.
5:1	2.5	0.42
5:3	1.8	0.07
6:1	1.2	0.04
6:3	1.5	0.14

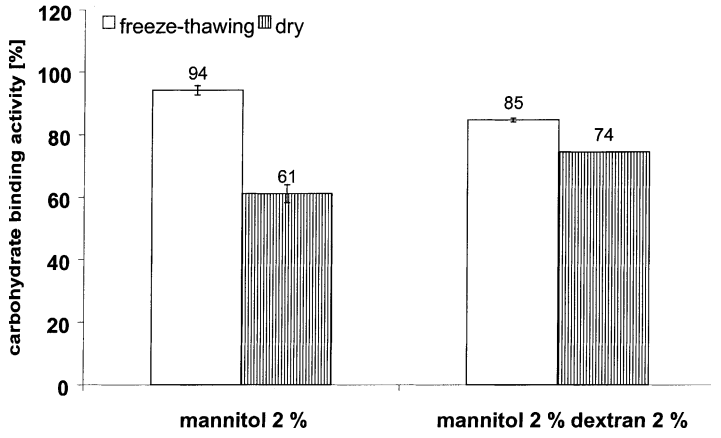

Fig. 6. Influence of mannitol on the activity of aviscumine freeze-dried in 100 mM Tris buffer, pH 8.0, with 0.1% Tween® 80 and dextran T1 ($n = 4$; S.D. = error bar).

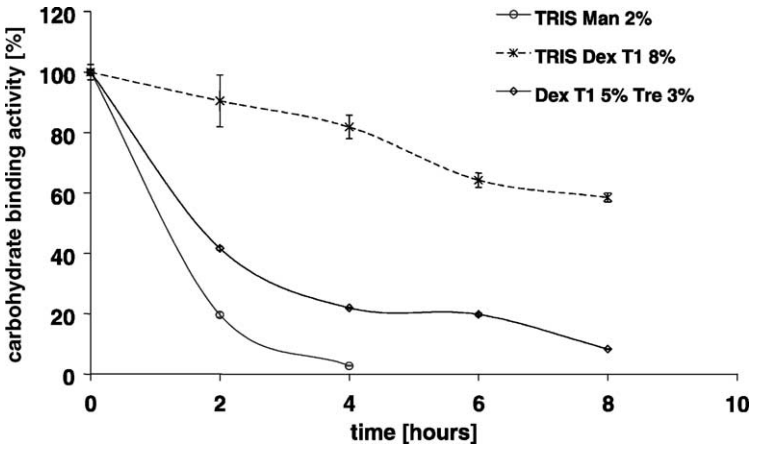

Fig. 7. Stability of freeze-dried aviscumine at elevated temperature (60 °C) ($n = 2$; S.D. = error bar).

not shown) showed a crystalline constitution for the dried product lyophilized with mannitol even in combination with dextran. The phase separation due to crystallization leads to protein degradation. Therefore, trehalose and also mannitol showed no advantage in the stabilization of aviscumine.

The results of stability studies at elevated temperature are presented in Fig. 7. The preparation of aviscumine in a buffer system with 2% mannitol showed a tremendous decrease in carbohydrate-binding activity of about 80% ($P \leq 0.00013$) after 2 h. If mannitol is replaced by 5% dextran T1 and 3% trehalose, the decrease in activity after 2 h is about 60% ($P \leq 0.00064$), and if only 8% of dextran T1 is used the activity of the protein is not reduced significantly (decrease of activity was about 8%, $P \leq 0.2719$). These results demonstrate the stabilization capability of low molecular weight dextrans.

4. Conclusion

The stabilization of aviscumine with low molecular weight dextrans was investigated in relation to concentration and molecular weight. A concentration of 8% (m/v) dextran T1 (1 kDa) in a 100 mM Tris buffer, pH 8.0, with 0.1% Tween® 80 recovered 90% of the carbohydrate-binding activity of

aviscumine after freeze-drying and reconstitution. This result is explained by both the ability of dextran to create an amorphous cake structure as well as protein−excipient interactions. Aviscumine is the first protein stabilized with an alkaline buffer system (pH 8), and it is interesting to know that stabilization theories derived from acid buffer systems can be transferred to alkaline buffer systems as well. The protein concentration itself did not influence the activity of aviscumine in preparations with 8% dextran. The addition of either trehalose or mannitol in combination with dextran did not enhance the stability of the protein. Stress stability data showed the extended effect of low molecular weight dextran on the stability of aviscumine in freeze-dried products.

Acknowledgements

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