ELSEVIER

International Journal of Pharmaceutics 159 (1997) 19–25

international journal of pharmaceutics

Stabilization of fully active chymopapain by lyophilization

Philippe M. Dekeyser [a,*], Sam Corveleyn [b], Joseph Demeester [a], Jean-Paul Remon [b]

[a] *Laboratories for General Biochemistry and Physical Pharmacy, University of Gent, Harelbekestraat 72, B-9000 Gent, Belgium*
[b] *Laboratory of Pharmaceutical Technology, University of Gent, Harelbekestraat 72, B-9000 Gent, Belgium*

Received 6 June 1997; accepted 13 August 1997

Abstract

The use of maltodextrins, partially hydrolysed starches, was evaluated for the stabilization of fully activated chymopapain, a cysteine proteinase from the papaya latex. Chymopapain was fully activated with cysteine and Na_2EDTA. Enzyme activity was determined before and after the stabilization of chymopapain by lyophilization, in the presence of different amounts of lyoprotectants. During 3 years, the stability of one of the formulations was evaluated. Differential Scanning Calorimetry (DSC) was used to determine the shelf temperature of these products, while X-ray diffraction was used to determine the crystallinity of the products. The water content was determined using Karl-Fisher titration. Maltodextrins were found to protect the fully active chymopapain during the lyophilization process. The protection was dependent on their dextrose equivalent (DE) value and independent on the concentration used (2–5% w/v). The degree of protection was as good as that obtained by sucrose. The activity of chymopapain, stabilized by maltodextrin DE 28, was constant when stored for 3 years at room temperature. Freeze-dried cakes of maltodextrin formulations, containing small amounts of cysteine and Na_2EDTA, were amorphous. Maltodextrins can be considered as potential lyoprotectants in the lyophilization of fully active cysteine proteinases. © 1997 Elsevier Science B.V.

Keywords: Lyophilization; Enzymes; Chymopapain; Maltodextrins

1. Introduction

Chymopapain, a cysteine proteinase of the papain family, was used as a model compound to test the possibility of stabilizing fully activated proteinases by lyophilization. Chymopapain is used in medicine for chemonucleolysis. Chemonucleolysis is the treatment of sciatica by the intradiscal injection of a chymopapain solution (Buttle et al., 1986). The pharmaceutical applications for this group of proteinases is complicated by their relatively poor stability in solution, especially when used in highly purified form. In solu-

* Corresponding author. Tel.: +32 9 2285283; fax: +32 9 2281059; e-mail: Philippe.Dekeyser@rug.ac be

PII S0378-5173(97)00265-2

tion, these enzymes are rapidly inactivated by both oxidation and autocatalytical degradation (Cockayne et al., 1985). On the other hand, these enzymes must be fully activated to obtain maximal activity. During the lyophilization process however, the folding of the enzyme could be altered, leading to a loss of enzyme function. Addition of crystalline molecules, such as cysteine and Na_2EDTA, required to obtain a fully activated form of the enzyme, could lead to a partially crystalline end product. The degree of crystallinity of such a product could increase upon storage, resulting in a increasing degradation during storage. As a protection for proteins and enzymes during the lyophilization process, different lyoprotectants have been tested. Additives, including sugars, polyols, amino acids and surfactants have been investigated as potential lyoprotectants and the mechanisms of action were discussed (Carpenter et al., 1991). Carbohydrates might protect dried proteins because these solutes bind to dried proteins, serving as a 'water substitute' when the hydration shell of the protein is removed (Carpenter and Crowe, 1989). Carpenter and Crowe reported that hydrogen bonding of a solute to the protein is found to be requisite for protein protection during freeze-drying (Carpenter et al., 1987). Maltodextrins are widely used in food industry to retard crystallization, to improve drying characteristics, to decrease hygroscopicity of dried food products and to improve storage stability of frozen foods (Levine and Slade, 1986). Corveleyn and Remon (1996) reported on the effectiveness of maltodextrins in the lyophilization of LDH as a model protein. Research on the immunogenecity should provide evidence for the use of maltodextrins as possible excipients in parenteral formulations. The objectives of this study were to evaluate the potential use of different maltodextrins for the stabilization of fully activated chymopapain by lyophilization.

2. Materials and methods

Chymopapain was purchased from United States Biochemicals (Amersham Belgium, Gent, Belgium). The spray dried maltodextrins (Eridania-Beghin Say-Cerestar, Vilvoorde, Belgium) were obtained by enzymatic hydrolysis of corn starch, and had different dextrose equivalents (DE): C*PUR01910 (DE = 12), C*PUR01921 (DE = 22), C*PUR01924 (DE = 28) and C*PUR01934 (DE = 38). The carbohydrate composition and molecular weight of these maltodextrins have been published previously (Corveleyn and Remon, 1996). Cysteine and Na_2EDTA were from Janssen Chimica (Beerse, Belgium). Sucrose was from Alpha-Pharma (Zwevegem, Belgium). Karl Fischer reagents used were Hydranal Composite 5 and dried methanol (Riedel de Haen, Seelze, Germany). All other reagents were of analytical grade or the best commercial grade available.

2.1. Biochemical characterization of chymopapain

The characterisation was performed essentially as described previously (Dekeyser et al., 1994). A total of 200 mg of chymopapain was loaded on a *S*-Sepharose HP column and eluted using a NaCl gradient. Alternatively, native acid electrophoresis was performed.

2.2. Assay for chymopapain activity

The enzyme assay was performed as described previously (Dekeyser et al., 1994). A total of 200 μl of each sample was added to 800 μl of 0.03 M citric acid-0.14 M disodium phosphate buffer, pH 6.4, containing 100 mM of cysteine, HCl and 1 mM Na_2EDTA. The enzyme was allowed to activate at 37°C for 5 min before the reaction was started, by addition of 750 μl substrate solution. The substrate solution was prepared by addition of 2 ml of DMSO to 50 mg N-α-benzoyl-L-arginine-*p*-nitroanilide hydrochloride (L-BAPA). Subsequently, this solution was diluted 1:50 in citrate-phosphate buffer. After 30 min, the reaction was stopped by addition of 250 μl of 60% (v/v) acetic acid. Released 4-nitroaniline was determined by measurement of the absorbance at 410 nm. One unit of activity (nkat) corresponds to the release of 1 nmol of 4-nitroaniline ($\varepsilon = 8800$ $M.cm^{-1}$) per s from L-BAPA at pH 6.4 and 37°C. Results are presented as mean $\pm$ S.D. ($n = 3$).

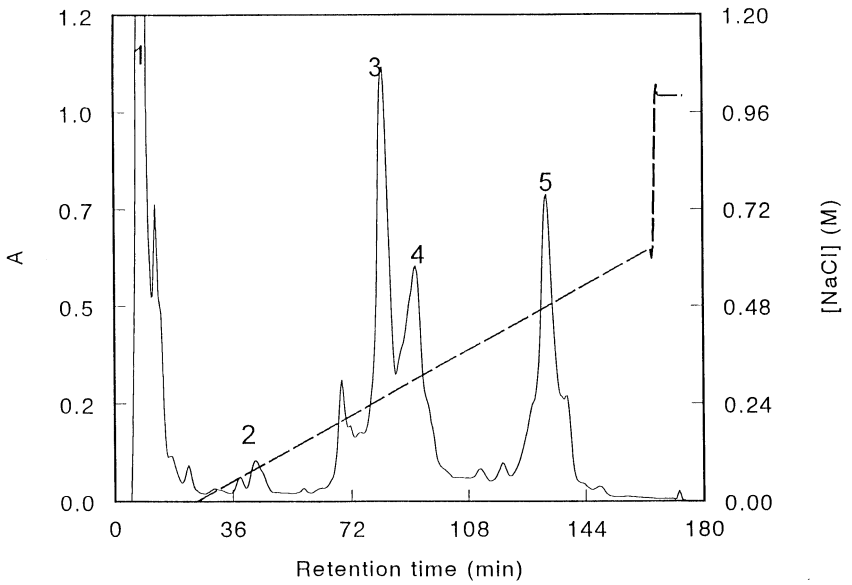

Fig. 1. Ion-exchange chromatography of the chemically blocked chymopapain (USB) on a HiLoad *S*-Sepharose HP 26/10 column. Column: 100 × 26 mm I.D.; fraction volume: 14.4 ml; flow-rate: 8 ml/min; buffer: 150 mM sodium acetate buffer pH 5.0; elusion with a linear gradient of sodium chloride from 0–625 mM in 140 min. (——): A_{280}; (— — —): sodium chloride gradient. Fraction 1: unbound material; fraction 2: papain; fraction 3: chymopapain A (degradation product); fraction 4: mixture of chymopapain and glycyl endopeptidase and fraction 5: caricain.

2.3. Lyophilization

Enzyme solutions containing 0.5 mg/ml chymopapain (final concentration) were lyophilized. The enzyme was activated by 0.375 mg/ml cysteine and 0.1 mg/ml Na_2EDTA. The enzyme solutions were mixed with the solutions of different maltodextrins or sucrose and 1 ml of the final solution was filled into 8 ml Type I glass vials (Gaash Packaging, Mollem, Belgium). Bromobutyl stoppers (Helvoet Pharma, Alken, Belgium) were partially inserted into the vials and the solutions were freeze-dried in an Amsco-Finn Aqua GT4 freeze-dryer. The samples were frozen on the lyophilizer shelves to −42°C in 2 h and were kept at this temperature for another 1 h. Primary drying was performed by keeping the vials for 14 h at a pressure of 0.1 mbar, a shelf temperature of −35°C and a condensor temperature of −60°C. Secondary drying was carried out by increasing the shelf temperature to 20°C at a pressure of 10 mbar. The secondary drying time was 6 h.

Lyophilization was terminated by venting the drying chamber with air and the vials were sealed by automatic stopping in the freeze-dryer. The samples were reconstituted by addition of 1 ml distilled water. The remaining activity was calculated as a percentage of the activity before lyophilization.

2.4. Differential scanning calorimetry (DSC)

The differential scanning calorimeter (DSC-2920, TA Instruments, Gent, Belgium) was calibrated using the melting transition of indium. Samples of the freeze-dried powders (ca. 5–20 mg) were placed in aluminium pans, non hermetically sealed and scanned at 10°C/min from 25 to 250°C. An empty sample container was used as the reference pan. The glass transition of the freeze-dried samples was calculated as the midpoint of the transition. Each sample was analysed in triplicate.

2.5. X-ray diffraction

The raw materials and freeze-dried samples were evaluated on crystallinity using X-ray diffraction (Diffractometer D5000, Cu, Kα, Siemens, Germany).

2.6. Determination of residual water content

The freeze dried samples were evaluated on residual water content using Karl Fischer titration (Mettler DL35, Mettler-Toledo, Lot, Belgium). The instrument was calibrated using disodium tartrate and water as a standard (Riedel de Haen, Seelze, Germany). The lyophilized cakes were pulverized and immediately analysed after a stirring time of 2 min. Results are presented as mean $\pm$ S.D. ($n = 3$).

3. Results and discussion

3.1. Biochemical characterization of chymopapain

USB chymopapain is available as a freeze-dried powder with a high protein content (77.4%), primarily balanced with thiamine pyrophosphate chloride as an excipient. The chymopapain solution was dialysed overnight at 4°C in order to remove the excipient before lyophilization. We could demonstrate, using ion-exchange chromatography and electrophoresis (results not shown), that USB chymopapain is a complex protein mixture, containing at least papain, chymopapain and caricain (Fig. 1). Using USB chymopapain as a starting material, all four known papaya proteinases, namely papain, chymopapain, caricain and glycyl endopeptidase, could be purified from a single batch of USB chymopapain.

Table 1
Influence of the type and concentration of maltodextrin on the activity recovery of chymopapain (0.5 mg/ml)

Type MD	Concentration excipient[a]			
	2% (w/v)	3% (w/v)	4% (w/v)	5% (w/v)
DE 12	80 ± 7	76 ± 1	77 ± 2	77 ± 3
DE 22	103 ± 5	100 ± 5	100 ± 3	95 ± 3
DE 28	103 ± 2	100 ± 1	101 ± 1	100 ± 2
DE 38	101 ± 2	100 ± 6	98 ± 1	101 ± 1

[a] Activities are expressed in percentage remaining activity after lyophilization

Table 2
Stability of freeze-dried activated chymopapain

Storage time (months)	Activity (%)[a]
0	100 ± 1
10	102 ± 1
22	102 ± 2
34	96 ± 2

Chymopapain (0.5 mg/ml), was stabilized with MD DE 28, at room temperature over a time period of 3 years.
[a] Results are mean $\pm$ S.E.M. ($n = 3$).

3.2. The effect of the maltodextrins on the activity recovery after lyophilization

The enzyme assay was validated and the activity was found to be linearly increasing with the enzyme concentration between 0–100 pkat. The relative S.D. was 1% ($n = 9$). This clearly shows that the determination of the chymopapain activity can be performed without the use of a chymopapain standard.

Table 1 shows the effect of four different maltodextrins on the activity recovery of chymopapain, immediately after lyophilization. Maltodextrins, as well as sucrose, protected the enzyme during freeze-drying and the activity recovery was dependent on the dextrose equivalent of the maltodextrin used. An activity recovery of more than 95% was obtained, using maltodextrins with DE > 22. We could not detect the physical properties leading to the loss of activity during lyophilization when the maltodextrin DE 12 was used as lyoprotectant. All products freeze-dried with full retention of structure.

There was no influence of the maltodextrin concentration when used in a range between 2–5% w/v. The relation between the glass transition temperature of a maltodextrin solution (Tg′) and the DE value has been reported previously (Levine and Slade, 1986). A possible advantage of

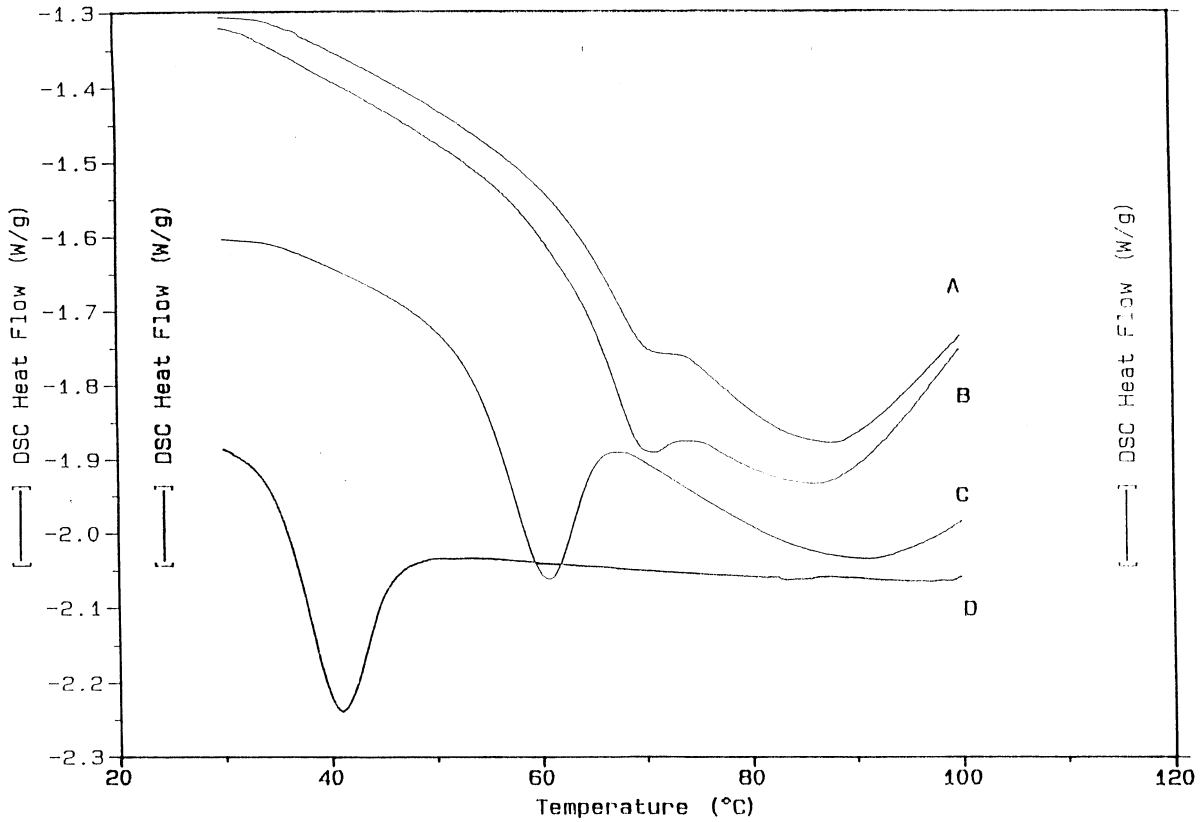

Fig. 2. DSC thermograms of freeze-dried chymopapain formulations (0.5 mg/ml) stabilized with different lyoprotectants (5% w/v) after a storage period of 3 years at room temperature: DE 22 (A); DE 28 (B) and DE 38 (C) and sucrose (D).

using low DE maltodextrins having a Tg′ above −15°C in lyophilization is the increase in product temperature that can be used during primary drying. Higher product temperatures could result in shorter lyophilization cycle times. X-ray diffraction of the freeze-dried chymopapain/maltodextrin formulations revealed the products to be amorphous. The amorphous character of the product was not affected by the addition of crystalline cysteine and Na_2EDTA to the formulation. Amorphism is known to be an essential condition in the stabilization of freeze-dried proteins (Izutsu et al., 1993). Maintenance of the amorphous state results in molecular interaction of lyoprotectants with the protein, which has been reported to be necessary for stabilising proteins during lyophilization (Carpenter and Crowe, 1989; Carpenter et al., 1991).

3.3. The long-term stability of fully activated chymopapain

The stability of activated chymopapain (0.5 mg/ml final concentration), stabilized with 5% w/v maltodextrin DE 28, and stored at room temperature was followed during a time period of 3 years. Results of this stability study are shown in Table 2. During this 3-year evaluation period, the relative activity of the sample was still $>96\%$ ($\pm$ 1%), indicating that only limited degradation of the product had occurred. Further proof for the stability of the product was obtained from the physical characterization of the freeze dried samples. X-ray diffraction patterns (results not shown) revealed that the samples remained amorphous even after the storage period of 3 years. The residual water content of the freeze-dried

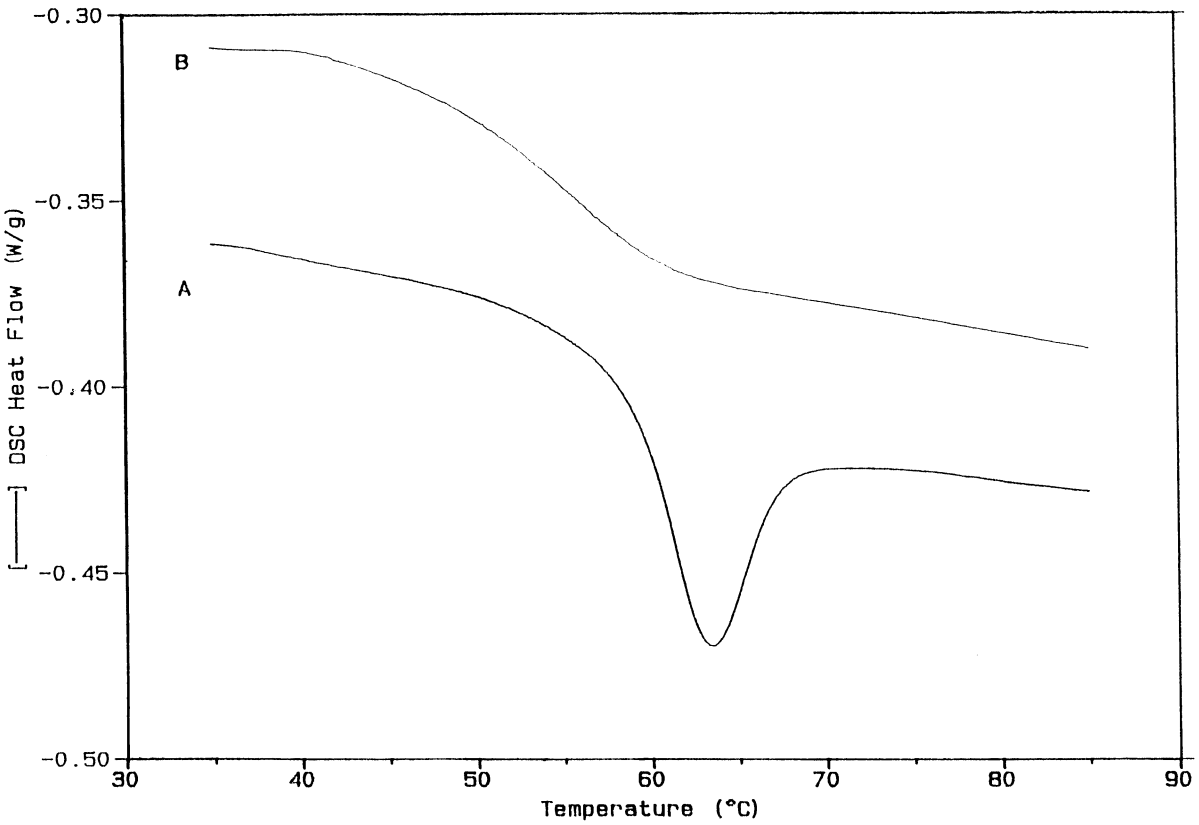

Fig. 3. DSC thermograms of freeze-dried chymopapain formulations (0.5 mg/ml) stabilized with DE 38 (5% w/v) after a storage period of 3 years at room temperature: first scan (A) and second scan (B).

cakes was between 2 and 3% (after 3 years). DSC thermograms of chymopapain freeze-dried with maltodextrins having different DE values are shown in Fig. 2. A glass transition temperature (Tg) was observed with an endotherm superimposed on this transition. The intensity of this endotherm increased with increasing DE value of the maltodextrin. The appearance of this peak could be due to physical aging which is a relaxation process at a temperature below Tg and is characterised by a change in thermal and mechanical properties. Roy et al. (1991) reported on the presence of an endotherm as a 'relaxation peak' superimposed on the glass transition of an amorphous lyophilized monoclonal antibody. The relaxation endotherm did not appear when the same sample was cooled and scanned a second time: a clear glass transition was seen (Fig. 3). This phenomenon was also observed by Hatley (1991).

The glass transition temperature (Tg) and moisture content of the freeze-dried samples as a function of DE value are summarized in Table 3. The freeze-dried chymopapain formulation, stabilized by sucrose (5% w/v) had a glass transition temperature of 38.5 ± 1.8°C. The glass transition temperature of the maltodextrin formulations was in the range 57.45–69.52°C and increased with a decreasing DE value. The Tg value of the dried products gives the maximal safe storage temperature of the formulation (Hatley and Franks, 1991). If the formulation is kept below Tg, the protein will be in an amorphous glass, physically protected from decay (Franks, 1990) The high Tg value of the formulations with low DE maltodextrins can be an additional advantage of the use of these excipients in freeze-dried preparations. Re-

Table 3
Water content and glass transition temperature (Tg) of freeze-dried chymopapain (0.5 mg/ml) in combination with sucrose and maltodextrins having different DE values

Formulation	Residual water content (%)	Glass transition (Tg)[a] (°C)
Sucrose (5% w/v)	2.5 ± 0.5	38 ± 2
Maltodextrin DE 12 (5% w/v)	2.3 ± 0.3	74.8 ± 0.7
Maltodextrin DE 22 (5% w/v)	2.5 ± 0.3	64.8 ± 0.8
Maltodextrin DE 28 (5% w/v)	2.4 ± 0.3	67 ± 1
Maltodextrin DE 38 (5% w/v)	2.7 ± 0.5	57 ± 2

[a] Calculated as the midpoint of the transition, mean ± S.D. ($n = 3$).

search on the immunogenecity should provide evidence of the use of these maltodextrins as possible excipients in parenteral formulations.

4. Conclusions

Maltodextrins were found to protect fully activated chymopapain during lyophilization. The protection was dependent on the DE value of the maltodextrins used and independent on the concentrations used in a concentration range from 2 to 5% (w/v). The amorphous character of the product was not affected by the inclusion of cysteine and Na_2EDTA in the preparation. Chymopapain activity in a freeze-dried formulation with maltodextrin DE 28 was constant, over a time period of 3 years at room temperature. Although comparable results can be obtained using sucrose as lyoprotectant, additional advantages can be obtained by the use of maltodextrins as lyoprotectants. Using low DE maltodextrins, higher product temperatures can be used during primary drying, resulting in shorter lyophilization times. The final products, stabilized by maltodextrins, had higher Tg values (57–69°C) than those stabilized by sucrose (38.5°C) indicating that they should be better protected against a temperature increase.

References

Buttle, D.J., Abrahamson, M., Barrett, A.J., 1986. The biochemistry of the action of chymopapain in relief of sciatica. Spine 11, 688–694.

Carpenter, J.F., Crowe, L.M., Crowe, J.H., 1987. Stabilization of phosphofructokinase with sugars during freeze-drying: Characterization of enhanced protection in the presence of divalent cations. Biochim. Biophys. Acta 923, 109–115.

Carpenter, J.F., Arakawa, T., Crowe, J.H., 1991. Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. Dev. Biol. Stand. 74, 225–239.

Carpenter, J.F., Crowe, J.H., 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28, 3916–3922.

Cockayne, D., Willatt, J.E., Buttle, D.J., Day, S., 1985. Relative activity and stability of commercially available chymopapain formulations for chemonucleolysis. In: Sutton, J.C. (Ed.), Current Concepts in Chemonucleolysis. Royal Society of Medicine, London, pp. 9–16.

Corveleyn, S., Remon, J.P., 1996. Maltodextins as lyoprotectants in the lyophilization of a model protein, LDH. Pharm. Res. 13, 146–150.

Dekeyser, P.M., De Smedt, S., Demeester, J., Lauwers, A., 1994. Fractionation and purification of the thiol proteinases from the papaya latex. J. Chromatogr. B. Biomed. Appl. 656, 203–208.

Franks, F., 1990. Freeze drying: From empericism to predictability. Cryo. Lett. 11, 93–110.

Hatley, R.H.M., 1991. The effective use of DSC in the optimization of freeze-drying processes and formulations. Dev. Biol. Stand. 74, 105–122.

Hatley, R.H.M., Franks, F., 1991. Application of DSC in the development of improved freeze-drying processes for labile biologicals. J. Therm. Anal. 37, 1905–1914.

Izutsu, K., Yoshioka, S., Terao, T., 1993. Decreased protein-stabilizing effects of cryoprotectants due to crystallization. Pharm. Res. 10, 1232–1237.

Levine, H., Slade, L.A., 1986. A polymer physico-chemical approach to the study of commercial starch hydrolysis products (SHPs). Carbohydr. Polym. 6, 213–244.

Roy, M.L., Pikal, M.J., Rickard, E.C., Maloney, A.M., 1991. The effects of formulation and moisture on the stability of freeze-dried monoclonal antibody-vinca conjugate: A test of the WLF glass transition theory. Dev. Biol. Stand. 74, 323–340.