# Hydroxypropyl-$\beta$-cyclodextrin Inhibits Spray-Drying-Induced Inactivation Of $\beta$-Galactosidase

SÉBASTIEN BRANCHU, ROBERT T. FORBES,* PETER YORK, SVEN PETRÉN,† HÅKAN NYQVIST,† AND OLA CAMBER‡

Contribution from *Drug Delivery Group, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK.*

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**Abstract** □ The single-step, fast spray-drying process may represent a valuable alternative to the multistep, time-consuming freeze-drying process in the area of formulation and processing of biopharmaceuticals. In this study, we tested the use of sucrose and hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD) as stabilizing excipients in the spray-drying of a model protein, $\beta$-galactosidase. The solutions were processed using a Büchi 190 cocurrent Mini Spray Dryer at an outlet temperature of $61 \pm 2$ °C. The powders were redissolved and analyzed for catalytic activity, aggregation, chemical decomposition, and thermal susceptibility as observed by high-resolution calorimetry. Spray-drying significantly inactivated $\beta$-galactosidase. Spray-drying $\beta$-galactosidase in the presence of sucrose did not prevent inactivation. However, after spray-drying $\beta$-galactosidase in the presence of HP-$\beta$-CD, or HP-$\beta$-CD and sucrose, full catalytic activity was exhibited on reconstitution. Furthermore, the reconstituted product was unchanged in terms of molecular weight, charge, and thermal stability. These findings are consistent with a hypothesis that the change responsible for inactivation of $\beta$-galactosidase was mainly a monomolecular, noncovalent change, i.e., the formation of incorrect structures, that arose from surface denaturation. This study clearly demonstrates that cyclodextrins can be useful stabilizing excipients in the preparation of spray-dried protein pharmaceuticals.

With the advent of new drug therapies and the increasing commercial availability of biotechnology-derived proteins, great expectations have arisen from the development of biopharmaceuticals.[1] However, a significant technical challenge lies in the formulation of these drugs. Compounds such as proteins are often physicochemically fragile and readily degrade,[2] the most common degradation pathways being aggregation, fragmentation, deamidation, and oxidation, which influence critical pharmaceutical properties including biological activity, metabolic half-life, and immunogenicity.[3] Freeze-drying has in general been the method of choice for stabilizing labile biopharmaceuticals, while spray-drying has been used for the stabilization of various heat-sensitive biological (enzymes), food (milk and egg products), and pharmaceutical (antibiotics and vaccines) products.[4] Despite the relatively high temperatures involved in the use of a hot airstream as a drying medium, the droplets in a spray-dryer do not attain a high temperature. Additionally, the low water content of the product that leaves the drying chamber allows the labile compound to withstand the effect of heat. Thus, spray-drying has been used under certain conditions to produce fully biologically active proteins.[5,6] The controllable particle size and shape characteristics of spray-dried powders make this process suited to the formulation of pharmaceuticals for inhalation.[7] Thus, the single-step, fast spray-drying process may represent a valuable alternative to the multistep, time-consuming freeze-drying process in the area of formulation and processing of biopharmaceuticals.

Nonetheless, the physical stress of the drying process on proteins may cause degradation,[3,8,9] which necessitates the use of stabilizing excipients when preparing dried biopharmaceuticals. Sugars and other polyols have been used as protein stabilizers because of their preferential interaction behavior in solution.[10] During spray-drying, polyols have been observed to reduce the rate constant of protein inactivation and improve recovered enzyme activity.[11,12] In contrast, cyclic oligosaccharides, or cyclodextrins (CDs), favor protein thermal denaturation in solution.[13] However, cyclodextrins have been reported to stabilize proteins against other protein degradation pathways, such as aggregation[14] and precipitation,[15] and as a result improve protein folding reversibility.[16] Cyclodextrins have also been shown to protect proteins from freeze-drying-induced inactivation[17] and maintain the native structure in elevated temperature stability studies.[3] Cyclodextrins are thought to have an impact on protein behavior through weak interactions between the oligosaccharide hydrophobic core and nonpolar protein groups when these are exposed.

Since polyols and cyclodextrins affect different protein degradation pathways, we wished to probe the influence of a combination of these two types of cosolvents on protein integrity during spray-drying and observe whether the two excipients would have additive, antagonistic, or synergistic effects, to compare these effects to those occurring in solution.[18] In this study, we tested the use of sucrose and hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD) as stabilizing agents in the spray-drying of a model enzyme, $\beta$-galactosidase derived from *Aspergillus oryzae*. A monomeric glycoprotein, *Aspergillus oryzae* $\beta$-galactosidase is a multidomain, 105 kDa, 4.5 pI enzyme.[19,20] In contrast to small, single-domain proteins, $\beta$-galactosidase may follow a two-step folding mechanism involving successively fast domain folding and slow domain pairing.[21] Unlike natural cyclodextrins, HP-$\beta$-CD is surface active;[22] it was chosen as a model cyclodextrin for its high aqueous solubility and its pharmaceutical relevance as a potential excipient for parenteral and oral routes.[23] Sucrose was chosen because of its good glass-forming abilities and its widespread use as a protein stabilizer. Powder samples were dissolved and analyzed for catalytic activity, aggregation, and chemical change. Further, high-sensitivity differential scanning calorimetry (HSDSC) was used to measure the susceptibility of the reconstituted enzyme to thermal denaturation. This technique measures directly the forces stabilizing the globular structure and has a potential predictive capability of long-term stability of proteins.[24]

* Corresponding author e-mail: R.T.Forbes@Bradford.ac.uk, fax +44 1274 234769, tel +44 1274 234653.

† Pharmaceutical Analysis, Analytical and Pharmaceutical R&D, Astra Arcus AB, S-151 85, Sodertalje, Sweden.

‡ Licensing Department, Astra Arcus AB, S-151 85, Sodertalje, Sweden.



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## Experimental Section

**Chemicals**—$\beta$-Galactosidase (EC 3.2.1.23) derived from *Aspergillus oryzae* (lot 115H1179), *o*-nitrophenyl-$\beta$-D-galactoside (ONPG, lot 105H5017), and anhydrous sucrose (lot 70H-018715) were obtained from Sigma-Aldrich Ltd, Poole, UK. Hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD, degree of substitution 0.6, batch 07B-271/1) was supplied by Janssen Biotech N. V., Olen, Belgium.

**Spray-Drying**—Each solution to be processed was prepared by dispersing 4 g of protein in 100 mL of deionized double-distilled water, with or without excipient, and centrifuged for 10 min at 13000 rpm. The concentration level was 1% w/v for each excipient, so that the sucrose:cyclodextrin:protein ratio was 0.25:0.25:1 in terms of mass and 76.8:19.0:1 in molar terms. A silicone tubing of inner diameter 4 mm and a peristaltic pump were used to feed the solutions to the nozzle of a Type B, Büchi 190 co-current Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland). This dryer has a two-fluid, pneumatic nozzle 0.5 mm in diameter. Cooling water was continuously run through the nozzle to prevent protein degradation in the nozzle. The solution feed rate was 0.36 $\pm$ 0.06 L $h^{-1}$, the air spray flow consumption 750 $\pm$ 50 L $h^{-1}$, and the aspirator vacuum level 10 (approximately 30000 L $h^{-1}$). The inlet and outlet temperatures were set to 190 $\pm$ 2 °C and 61 $\pm$ 2 °C respectively, because these temperatures were expected to cause some inactivation of *Aspergillus oryzae* $\beta$-galactosidase.[12]

**Catalytic Assay**—the samples were reconstituted in 100 mM sodium acetate buffer, pH 4.5, at an approximate protein concentration of 1 mg $mL^{-1}$, and yielded clear solutions. One hundred microliter aliquots of this solution were added to 3 mL of 4.93 mg.$mL^{-1}$ ONPG substrate solution. The rate of increase in absorbance at $\lambda$ = 420 nm and 37 °C, caused by the enzyme-catalyzed hydrolysis of ONPG to *o*-nitrophenol, was monitored for 1 to 2 min.[12] The relative standard deviation of this method is 5%. Protein determination was carried out by the Bradford (Coomassie blue) method at $\lambda$ = 590 nm, as described by Stoscheck.[25]

**High-Sensitivity Differential Scanning Calorimetry (HSDSC)**—Powders were dissolved in deionized double-distilled water to a protein concentration of 2−3 mg $mL^{-1}$. The solutions exhibited no turbidity and had a measured pH of 6.5, indicating that none of the excipients used affected the pH significantly. Thermal denaturation profiles for $\beta$-galactosidase were generated over the temperature range 20−100 °C using a Micro Calorimetry System high-sensitivity differential scanning calorimeter (MCS DSC, MicroCal Inc., Northampton, MA) controlled by the MicroCal Origin software. The instrument was calibrated for heat capacity using electrical pulses of known power and for temperature using sealed paraffin hydrocarbon standards of known melting points (28.2 °C and 75.9 °C for *n*-octadecane and n-hexatriacontane, respectively). Typically, this instrument has a noise level of 0.3 $\mu$W, a reproducibility on refilling the cells of 0.13 mJ $K^{-1}$, and a relative error in the heat capacity determination of 0.005%.[26]

The accuracy for the temperature measurement was 0.2 °C. All solutions were degassed for 2 min under vacuum before being loaded into the calorimeter, and were held under 2 bar nitrogen excess pressure to avoid bubble formation at higher temperatures. For each sample, a reference profile, obtained from water samples, was subtracted from the protein thermal data, and the excess heat capacity function was normalized for protein concentration. Since several processed samples contained sucrose or/and HP-$\beta$-CD, controls made of unprocessed protein and sucrose and/or HP-$\beta$-CD (in the same proportions as for processed samples) were prepared and analyzed in the same conditions. Thus, any change occurring both in the processed samples and in the controls would result from an excipient effect during the analysis and not during the spray-drying process.

**High-Performance Size-Exclusion Liquid Chromatography (HPSEC)**—For the chromatographic and electrophoretical experiments, the water used for reconstitution was deionized and filtered in a Milli-Q system (Millipore), and the samples were reconstituted in 0.1% aqueous acetic acid. Size-exclusion fractionation was performed in 50 mM phosphate buffer saline pH 7 at 40 °C using a TSKgel Super SW3000 column from TosoHaas GmbH (Stuttgart, Germany) made of 4 $\mu$m silica particles. This column was setup on a Merck-Hitachi HPLC chromatograph comprising an AS-2000 autosampler, a L-7100 pump, a LiChrograph L-6000 A HPLC pump, a D-6000 A interface, an L-5025 column thermostat, an L-4250 UV−vis detector set to $\lambda$ = 214 nm, and a water-circulated, temperature-controlled rack at 8 °C for the sample vials. Typically, duplicate samples (5 $\mu$L) were analyzed at a concentration of 1 mg/mL and using a flow rate of 0.35 mL/min. The calibration kit from BioRad contained thyroglobulin (670 kDa), bovine $\gamma$-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa). The data were analyzed using the Merck-Hitachi D-7000 HSM Chromatography Data Station Software.

**Table 1—Enzymatic Activity of Redissolved Spray-Dried $\beta$-Galactosidase Samples Relative to the As-Received, Commercial Protein[a]**

| | relative activity (%) |
|---|---|
| no excipient | 75.0 |
| sucrose[b] | 71.5 |
| HP-$\beta$-CD[b] | 98.3 |
| HP-$\beta$-CD:sucrose[b] | 90.0 |

[a] Reconstituted at a concentration of 1 mg $mL^{-1}$ in 100 mM sodium acetate buffer, pH 4.5. [b] One gram of each excipient was added to the 4% w/v protein solution (100 mL) prior to spray-drying.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Isoelectric Focusing (IEF)**—All materials were obtained from Amersham Pharmacia Biotech, Uppsala, Sweden, unless specified. Electrophoretical experiments were performed at 10 °C on a Multiphor II unit, with a thermostatic circulating water bath. SDS−PAGE runs were controlled by a BioRad 1000/500 power supply, and IEF ones by an Electrophoresis Power Supply EPS 3500 XL. The samples (run in quadruplicate) were separated according to molecular weight in both reducing and nonreducing conditions on ExcelGel SDS Homogeneous 12.5% containing 120 mM Tris, 120 mM acetate, 1 g $L^{-1}$ SDS, pH 6.4, and using ExcelGel SDS buffer strips at the anode and cathode made of 450 mM Tris-acetate, 4 g $L^{-1}$ SDS, pH 6.6, and 80 mM Tris, 800 mM Tricine, 6 g $L^{-1}$ SDS, pH 7.1, respectively. A high molecular weight calibration kit was used, for the range 36 kDa (beef heart lactate dehydrogenase subunit) up to 330 kDa (hog thyroid thyroglobulin subunit). After fixing, the gels were stained using a Coomassie, PhastGel Blue R solution. IEF experiments were carried out in the pH range 2.5−8.0, with prior rehydration of CleanGel in a mixture of 700 $\mu$L of Pharmalyte carrier ampholytes in the pH range 2.5−5.0 and 700 $\mu$L Pharmalyte pH 5.0−8.0. The IEF calibration kit covered the pH range 2.80 (pepsinogen) to 6.55 (human carbonic anhydrase B). The gels were stained with a 1 g $L^{-1}$ silver solution. All staining was done with an automated gel stainer (Hoefer Pharmacia Biotech Inc., San Francisco, CA).

## Results and Discussion

**Catalytic Data**—The enzymatic activity data measured for the reconstituted samples are given in Table 1. As expected from the choice of the processing conditions, and in particular the outlet temperature which is the main factor influencing recovered protein activity,[27,28] the reconstituted spray-dried *Aspergillus oryzae* $\beta$-galactosidase had a significantly lower catalytic activity (75%) than the as-received, commercial protein. Interestingly, the addition of 1% w/v sucrose in the feed protein solution did not significantly alter the activity of the reconstituted product. Broadhead et al.[12] have found sucrose to stabilize $\beta$-galactosidase during spray-drying. A noticeable difference in the conditions, though, was the sucrose:protein mass ratio, which was more than or equal to 1.67 in Broadhead et al.'s study, but as low as 0.25 in our work. In terms of inactivation kinetics, a level as low as 1% w/v sucrose would not slow the spray-drying-induced deterioration of glucose oxidase.[29] However, sucrose concentrations as low as that used here (0.03 M) have been found to stabilize oxyhemoglobin on spray-drying.[6] In contrast, our results show that the use of 1% w/v HP-$\beta$-CD fully protected $\beta$-galactosidase against inactivation. This is interesting as high concentrations (10 to 20%)[3] of this cyclodextrin are usually needed

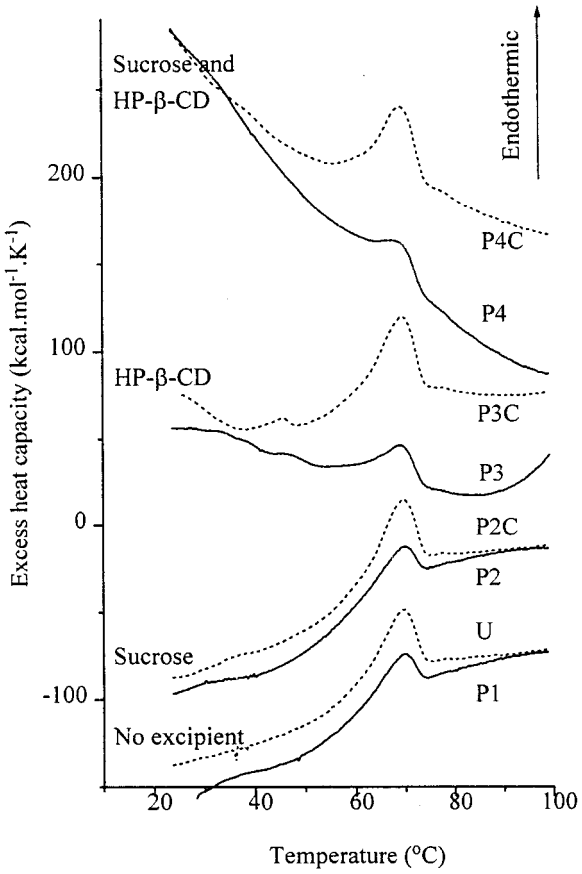


**Figure 1**—Thermal denaturation profiles for unprocessed (broken lines) and spray-dried (solid lines) $\beta$-galactosidase samples reconstituted in water at 2–3 mg mL$^{-1}$ protein concentration, pH 6.5. U: excipient-free, unprocessed; P1: excipient-free, processed; P2: sucrose-containing, processed; P2C: control for P2 (unprocessed enzyme to which sucrose was added); P3: HP-$\beta$-CD-containing, processed; P3C: control for P3; P4: sucrose- and HP-$\beta$-CD-containing, processed; P4C: control for P4. The excipient composition was matched for each pair of unprocessed/processed samples, as explained in the Experimental Section.

to affect protein stability. The recovered activity (90%) obtained through the use of a combination of both excipients did not differ significantly from that measured for the cyclodextrin-containing sample. Since these carbohydrates at such levels do not interfere with the measurement of enzyme kinetics,[18] it appears that the cyclodextrin used prevented spray-drying-induced inactivation of $\beta$-galactosidase, whereas sucrose did not provide any protection. This inactivation process and the excipient effects were further investigated with thermal and biochemical measurements.

**Thermal Denaturation Data**—The calorimetric profiles and derived thermodynamic parameters are given in Figures 1 and 2 and Tables 2 and 3. The calorimetric profile for commercial $\beta$-galactosidase displayed a single endotherm with a transition temperature, $T_m$, of 69.7 °C. All samples exhibited a similar profile and $T_m$ values were in the range 68.4−70.0 °C. The differences in slope were presumably due to excipient effects, as seen from the control data, and caused the small variations seen in $T_m$. Differences in transition enthalpies were ascribed to differences in concentrations of the reconstituted samples. All profiles were explained by a similar three-transition, non-two-state model. Differences were found in the relative intensities of the fitted transitions, but no trend appeared. The van't Hoff enthalpy, calculated for each fitted transition under the two-state approximation, yielded calorimetric-to-van't Hoff enthalpy ratios, which can be valuable for

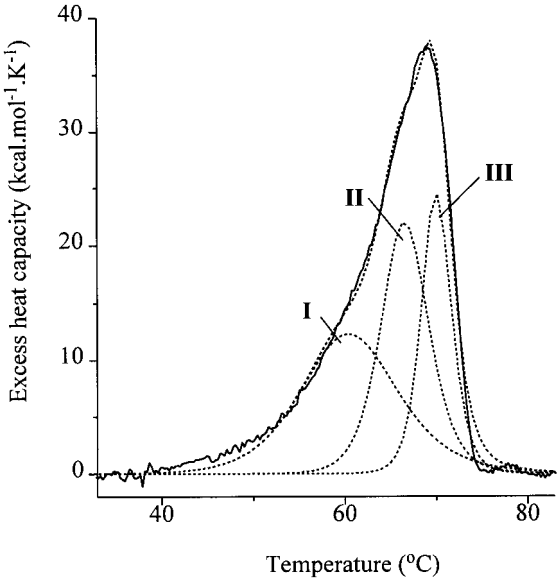


**Figure 2**—Three-transition, non-two-state model fitted to the calorimetric profile for reconstituted unprocessed $\beta$-galactosidase.

**Table 2—Observed Denaturation Temperatures ($T_m$) for Redissolved Unprocessed and Spray-dried $\beta$-galactosidase Samples**[a]

| | denaturation temperatures, $T_m$ (°C) | |
|---|---|---|
| | unprocessed | spray-dried |
| no excipient | 69.7 | 70.0 |
| Sucrose[b] | 69.7 | 69.9 |
| HP-$\beta$-CD[b] | 69.5 | 69.1 |
| HP-$\beta$-CD:sucrose[b] | 69.0 | 68.4 |

[a] The excipient:protein mass ratio was 0.25 for each excipient in all preparations. [b] One gram of each excipient was added to the 4% w/v protein solution (100 mL) prior to spray-drying.

estimating the number of cooperative units. Transition I generally exhibited ratios much larger than 1, except for the HP-$\beta$-CD sample (P3). Transition II yielded ratios in the interval 0.6−1.1. Transition III showed ratios significantly less than 1, except for the unprocessed sample (U). However, because of the number of factors (e.g., baseline determination, exothermic processes, transition reversibility) that may bias the apparent enthalpies, no conclusions could be drawn from the observed calorimetric-to-van't Hoff enthalpy ratios.

**Chromatographic and Electrophoretical Profiles**—A representative size-exclusion HPLC trace is shown in Figure 3, with estimated parameters for all samples listed in Table 4. Electrophoretical fractions and estimates, respectively, are given in Figures 4 and 5 and Table 5. Fractionation by HPLC indicated the presence of five separated species, although not fully resolved. They were ascribed to the monomer (143 kDa), two fragments (53 and 42 kDa), a dimer (292 kDa), and a higher-MW aggregate (509 kDa). The peaks at 4.9 and 11.7 min were a high-molecular weight species eluting outside the size limits of the column and the buffer acetate anion, respectively. No effect of the spray-drying process or excipients on the retention times of these fractions was detected, although the relative intensity of the 9.0 min fragment peak was slightly larger for the sucrose sample (P2). Separation with SDS−PAGE of commercial $\beta$-galactosidase gave estimates for the main species of 105 kDa in nonreducing conditions and 119 kDa in the presence of mercaptoethanol. This band was attributed to the monomer. Additionally, three main bands appeared with MW estimates of 70, 64, and 40 kDa, which were probably responsible for the 8.7 min and 9.0

**Table 3—Calorimetrically Derived Thermodynamic Parameters for Redissolved Unprocessed and Spray-Dried $\beta$-Galactosidase Samples[a]**

| sample | transition | $T_m$,[b] °C | $\Delta H_{cal}$,[c,f] kcal mol$^{-1}$ | $\Delta T_{1/2}$,[d] °C | $\Delta H_{vH}$,[e,f] kcal mol$^{-1}$ | $\Delta H_{cal}/\Delta H_{vH}$ |
|---|---|---|---|---|---|---|
| U | I | 60.3 | 172 | 12.2 | 72 | 2.38 |
| U | II | 66.4 | 153 | 6.1 | 150 | 1.02 |
| U | III | 68.6 | 192 | 6.1 | 152 | 1.26 |
| P1 | I | 60.1 | 121 | 11.2 | 79 | 1.54 |
| P1 | II | 66.5 | 98 | 6.0 | 153 | 0.64 |
| P1 | III | 70.1 | 57 | 3.6 | 260 | 0.22 |
| P2 | I | 59.5 | 134 | 12.5 | 70 | 1.91 |
| P2 | II | 66.2 | 98 | 6.3 | 146 | 0.67 |
| P2 | III | 69.8 | 59 | 4.0 | 232 | 0.25 |
| P2C | I | 61.9 | 183 | 11.5 | 77 | 2.36 |
| P2C | II | 67.7 | 168 | 5.3 | 175 | 0.96 |
| P2C | III | 70.6 | 84 | 3.4 | 279 | 0.30 |
| P3 | I | 60.7 | 88 | 8.3 | 107 | 0.82 |
| P3 | II | 66.6 | 103 | 5.9 | 156 | 0.66 |
| P3 | III | 70.0 | 79 | 4.2 | 225 | 0.35 |
| P3C | I | 62.1 | 148 | 9.7 | 92 | 1.61 |
| P3C | II | 67.3 | 188 | 5.7 | 162 | 1.16 |
| P3C | III | 70.6 | 103 | 3.7 | 257 | 0.40 |
| P4 | I | 60.9 | 170 | 11.3 | 78 | 2.17 |
| P4 | II | 67.0 | 134 | 6.1 | 150 | 0.90 |
| P4 | III | 70.3 | 63 | 4.3 | 221 | 0.29 |
| P4C | I | 62.4 | 203 | 10.1 | 88 | 2.29 |
| P4C | II | 67.7 | 194 | 5.3 | 175 | 1.11 |
| P4C | III | 70.7 | 90 | 3.5 | 267 | 0.34 |

[a] Abbreviations as in Figure 1. [b] Temperature of the maximum of the heat capacity function for each fitted transition. [c] Enthalpy change upon denaturation measured by integrating each transition. [d] Temperature width of the transition at half-height. [e] Van't Hoff enthalpy change calculated as $\Delta H_{vH} = 4RT_m^2/\Delta T_{1/2}$. [f] 1 cal = 4.184 J.

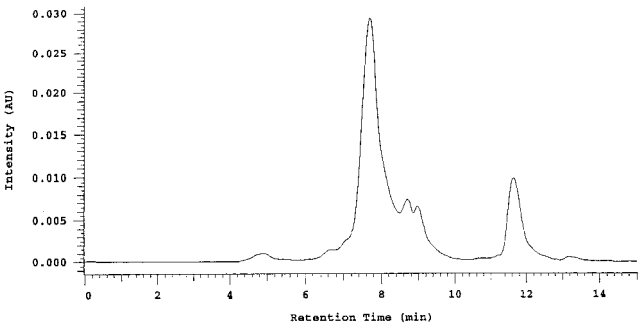


**Figure 3**—Size-exclusion HPLC profile observed for all unprocessed and spray-dried $\beta$-galactosidase samples reconstituted at 1–2 mg mL$^{-1}$ protein concentration in 0.1% aqueous acetic acid.

min HPLC peaks. The use of the surfactant SDS for gel electrophoresis allowed the nature of the aggregates detected with HPLC to be tested. The absence of electrophoretical band above the monomer-size band indicated that the high-molecular weight chromatographic fractions reflected noncovalent aggregates. Faint SDS−PAGE bands appeared in the range 29−35 kDa in reducing conditions, indicative of the presence of multichain species held by disulfides in nonreducing conditions. No spray-drying- or excipient-induced change in the molecular weight or relative amount of protein fractions was found. Isoelectric focusing of unprocessed $\beta$-galactosidase revealed a number of bands, of which the two main bands were in the 4.5 and 3.5 pI regions, consistent with the expected value. Neither the process nor the excipients altered the isoelectric profiles.

**Nature of the Inactivation Process and Cosolvent Effects**—Thus, spray-drying significantly inactivated $\beta$-galactosidase as observed by the level of recovered enzyme kinetic activity. The addition of HP-$\beta$-CD stabilized the enzyme against inactivation, as did the combination of both

**Table 4—Measured Retention Times (RT), Estimated Molecular Weights (MW), and Relative Areas for the Size-Exclusion HPLC Fractions of Unprocessed and Spray-Dried $\beta$-Galactosidase Samples[a]**

| | retention time, RT (min) | | | | | | relative area (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| sample | peak 1 | peak 2 | peak 3 | peak 4 | peak 5 | sample | peak 3 | peak 4 | peak 5 |
| U | ca. 6.7 | ca. 7.1 | 7.69 | 8.70 | 8.97 | U | 94.3 | 2.6 | 3.1 |
| P1 | ca. 6.7 | ca. 7.1 | 7.69 | 8.69 | 8.97 | P1 | 95.4 | 1.3 | 3.2 |
| P2 | ca. 6.7 | ca. 7.1 | 7.69 | 8.71 | 8.99 | P2 | 94.0 | 1.9 | 4.1 |
| P4 | ca. 6.7 | ca. 7.1 | 7.69 | 8.70 | 8.98 | P4 | 95.3 | 1.5 | 3.2 |
| P3 | ca. 6.7 | ca. 7.1 | 7.69 | 8.70 | 8.98 | P3 | 95.6 | 1.4 | 3.1 |
| P2C | ca. 6.7 | ca. 7.1 | 7.69 | 8.70 | 8.97 | P2C | 95.2 | 1.6 | 3.2 |
| P4C | ca. 6.7 | ca. 7.1 | 7.69 | 8.69 | 8.97 | P4C | 95.4 | 1.4 | 3.2 |
| P3C | ca. 6.7 | ca. 7.1 | 7.69 | 8.69 | 8.97 | P3C | 95.3 | 1.5 | 3.3 |
| MW | 509 | 292 | 143 | 53 | 42 | | | | |

[a] Abbreviations as in Figure 1. Relative areas were calculated without taking into account the 4.9 and 11.7 min peaks, which are a higher-molecular weight species eluting outside the size limits of the column, and the buffer acetate anion, respectively. Peaks 1 and 2 were too poorly resolved for meaningful integration.

HP-$\beta$-CD and sucrose, but not sucrose alone. These results demonstrate that cyclodextrins can be useful stabilizers in the preparation of spray-dried protein pharmaceuticals. Further experiments indicated that the spray-dried enzyme was unchanged in terms of thermal stability, size-exclusion HPLC, SDS−PAGE, and IEF patterns. These findings highlight the relevance of functional probes for the detection of certain changes in multidomain proteins that involve interactions between distant sites which remain undetected by physical probes.[21] The reduction in the activity that was observed may reflect either an almost complete loss of activity affecting only a fraction of the population of molecules, or a conformational alteration inducing less activity in most of the molecules.[30] Our data indicate that the structural change causing inactivation was subtle; it is likely that this change was not the formation of covalent or noncovalent aggregates, fragmentation, or deamidation. Since the droplets of a solution atomized in a cocurrent spray-drier usually reach a maximum temperature which is approximately 15 °C below the outlet temperature for 5 to 30 s,[11,31] $\beta$-galactosidase was only briefly exposed to temperatures approaching 45 °C, thus undergoing only small and reversible thermal denaturation as inferred from the calorimetric data. Temperatures in this region for a formulation at a pH close to neutral also mean that the inactivation process involved no change in primary structure.[32] Therefore, it is hypothesized that the change responsible for partial inactivation of $\beta$-galactosidase was mainly a monomolecular, noncovalent change, i.e., the formation of incorrect structures, and did not affect the thermal susceptibility of the protein but weakened its kinetic properties, i.e., interferred with its active site.

Furthermore, it is known that protein adsorption to surfaces can be a source of destabilization,[33] inducing, for example, inactivation of *Escherichia coli* $\beta$-galactosidase.[34] In our study, denaturation at the air−liquid interface was a probable explanation for inactivation because it was consistent with three relevant factors: the consistency between the absence of effect of sucrose and the expected large droplet surface coverage of the protein; indeed, it has been found that for a protein:lactose ratio of 1/99, the protein can dominate the droplet surface due to its higher surface activity than the sugar;[35] the reduction of inactivation in the presence of surface-active cyclodextrin, which may compete with protein molecules at the droplet surface; and the results of atomization studies indicating surface denaturation at the air−liquid interface of droplets in a spray and reduction of this instability by addition of

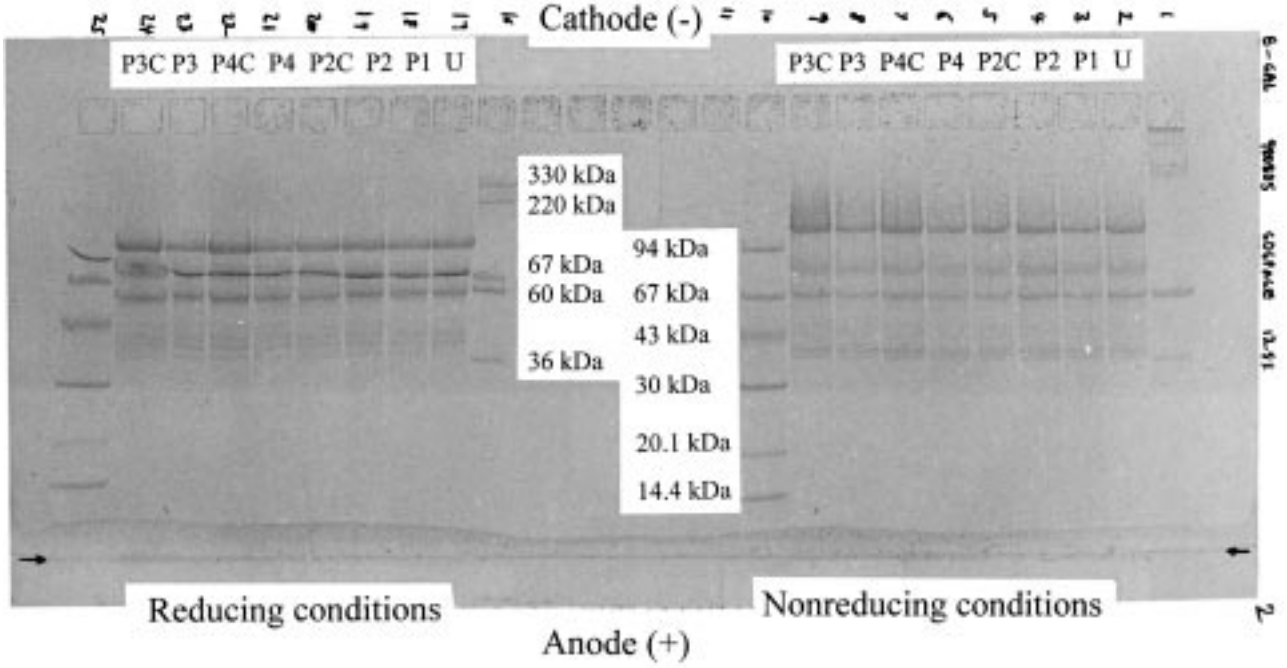


**Figure 4**—SDS–PAGE profile for unprocessed and spray-dried $\beta$-galactosidase samples. Same reconstitution conditions as in Figure 3. Reducing (left) and nonreducing conditions (right). Abbreviations as in Figure 1.

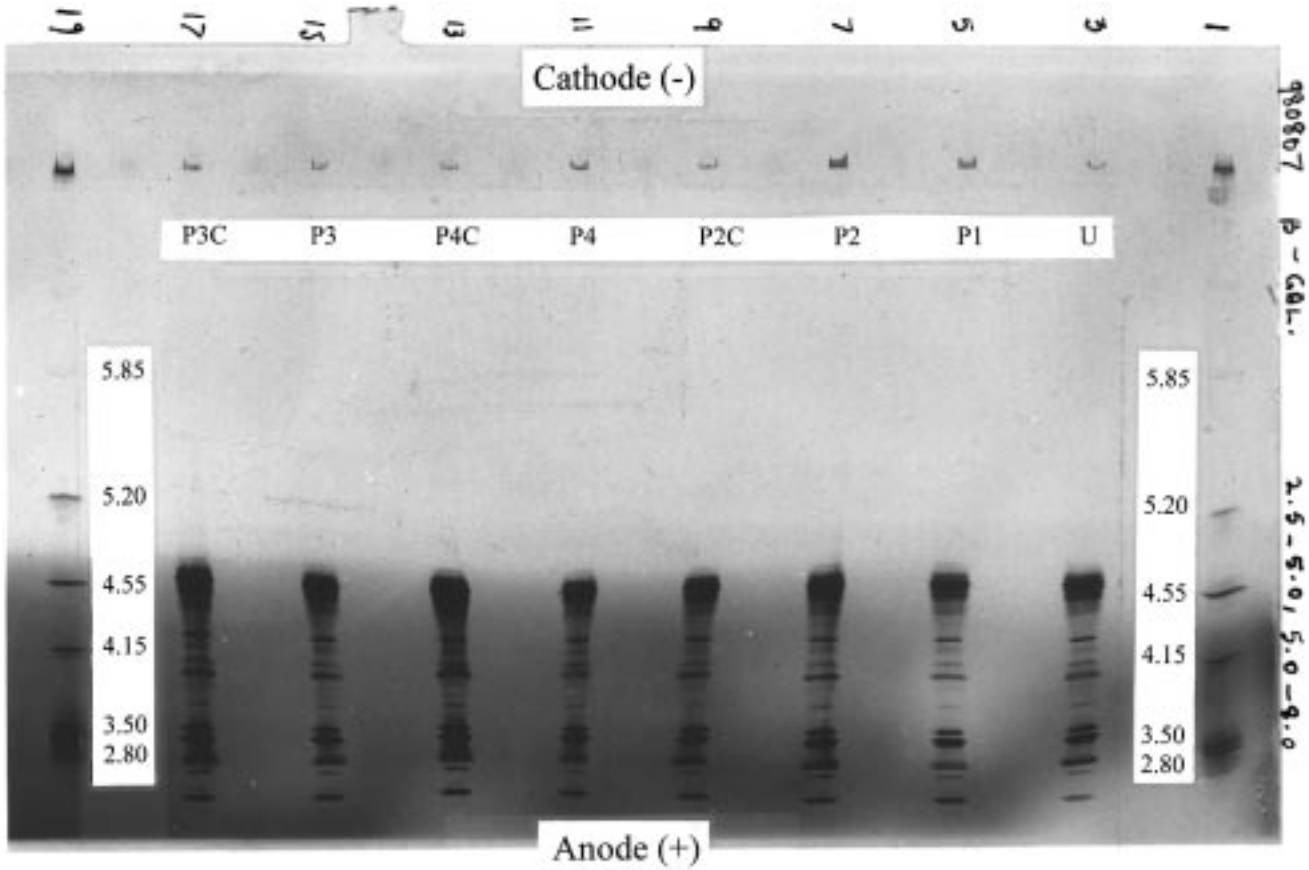


**Figure 5**—Isoelectric focusing (IEF) profile of unprocessed and spray-dried $\beta$-galactosidase samples. Same reconstitution conditions as in Figure 3. Abbreviations as in Figure 1.

surfactants.[5] Foaming that has been observed to result from the shear forces occurring during atomization[36] is another phenomenon that could explain the inactivation of $\beta$-galactosidase.

Thus, we propose that, in the particular formulation and processing conditions examined, i.e., at low outlet temperatures and low excipient:protein ratios, thermal degradation was small, and the usual thermodynamic stabilization provided by sucrose[29] did not occur to a significant extent; rather, it was primarily surface denaturation that caused inactivation, and HP-$\beta$-CD inhibited this instability pathway. It is further proposed that HP-$\beta$-CD may impact

**Table 5. —Estimated Molecular Weights (MW) and Isolectric Points of the Observed SDS–PAGE and IEF Fractions, Respectively, for Unprocessed and Processed β-Galactosidase Samples[a]**

| molecular weight (kDa) | | |
|---|---|---|
| nonreduced[b] | reduced[c] | isoelectric point |
| 105 | 119 | 4.62 |
| 78 | 89 | 4.47 |
| 63 | 64 | 4.25 |
| 42 | 38 | 4.05 |
| 41 | 35 | 3.95 |
| | 32 | 3.55 |
| | 29 | 3.40 |
| | | < 2.80 |

[a] No connection was established between molecular weights and isoelectric points of the fractions. [b] Nonreducing conditions. [c] In the presence of mercaptoethanol.

protein stability during spray-drying (i) by weak hydrophobic interaction between the oligosaccharide core and the protein nonpolar groups exposed by instability, hence inhibiting undesirable protein−protein and protein-interface interactions and (ii) by changing water properties since HP-β-CD is surface-active. The first of these mechanisms is partly cyclodextrin-specific, and may occur with nonderivatized cyclodextrins of different core dimensions, such as α-cyclodextrin,[13] but is also nonspecific, since any additives masking exposed nonpolar regions should prevent protein−interface interactions.

We believe that, depending on the processing conditions, spray-drying of proteins gives rise to a delicate balance between two instability pathways: thermal degradation and surface denaturation. Thermal degradation occurs in the interior of droplets due to the high water content in this region, but less at the droplet surface where there is less water.[37] It is consistent with the finding that thermal degradation is lower with smaller droplet size.[38] However, for surface denaturation at the droplet−air interface, it may be the opposite: it takes place at the surface and not in the interior, and it increases as droplet size decreases. That is why the ideal spray-dried formulation may necessitate a combination of differents excipients, such as a preferentially excluded stabilizer against thermal denaturation, and a surfactant against interfacial denaturation. Because of the number of effects involved, a multifactorial approach using factorial and/or composite designs[12,18] seems adequate.

In this study, oxidation was initially expected to be another possible degradation pathway.[29] This was not directly tested, e.g., through the use of dithiothreitol. However, the full enzymatic activity found for the cyclodextrin-containing formulation showed that if any oxidation was occurring, it did not affect the activity level of the formulation.

Besides the spray-drying process, the reconstitution step has to be considered explicitly. Aggregation is often observed to take place when dried proteins are reconstituted. It has been found that there may be differences in amount of aggregation based on additives in the initial solution as well as in the reconstitution medium.[39] In our study, the positive cyclodextrin effect might result from the ability of this excipient to prevent aggregation during the redissolution step. Additionally, if refolding occurred on reconstitution, then the presence of cyclodextrin would favor refolding because it would inhibit the off-pathway of aggregation. Overall, cyclodextrins may behave like naturally occurring chaperones that have similar functions in vivo.[40] From this viewpoint, the fast spray-drying process is attractive because, unlike freeze-drying, it occurs at nonequilibrium conditions, thus making less likely the achievement of a thermodynamically stable denatured state.[3] Therefore, on reconstitution, aggregation may not occur before refolding. In any case, our fractionation data suggested no aggregation, hence suggesting that the cyclodextrin effect took place during the process and not on reconstitution.

## Conclusions

We used sucrose and hydroxypropyl-β-cyclodextrin (HP-β-CD) as stabilizing agents in the spray-drying of a model enzyme, β-galactosidase. Spray-drying without stabilizing agents significantly inactivated β-galactosidase. The reconstituted samples containing HP-β-CD exhibited full catalytic activity, but not those containing sucrose as the sole stabilizing agent. All samples exhibited similar thermal profiles and transition temperatures. No general effect of the spray-drying process or excipients was detected on the retention times of the protein chromatographic fractions, although the relative intensity of the 9.0 min fragment peak was slightly larger for the sucrose-containing sample. No spray-drying- or excipient-induced change in the molecular weight or relative amount of protein electrophoretical fractions was found. These findings are consistent with a hypothesis that the change responsible for inactivation of β-galactosidase was a subtle monomolecular, noncovalent change, i.e., the formation of incorrect structures, which arose from surface denaturation and weakened the catalytic properties of the protein, i.e., interfered with its active site. The data suggest that the cyclodextrin inhibited protein denaturation by preventing adsorption at the droplet−air interface. This stabilization mechanism occurred mainly during the process rather than on redissolution. Sucrose did not provide any stabilization, presumably because of the nonthermal nature of the stress.

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