Aggregation of rhDNase Occurred During the Compression of KBr Pellets Used for FTIR Spectroscopy

Hak-Kim Chan,[1,2,3] Boonsri Ongpipattanakul,[1] and Jacky Au-Yeung[1]

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Purpose. To determine if a protein changes when it is compressed into a KBr pellet for FTIR spectroscopy measurement in the solid state, using recombinant human deoxyribonuclease I (rhDNase) as an example.

Methods. Lyophilized rhDNase with KBr compressed at different pressures were analyzed by FTIR spectroscopy, size exclusion HPLC and enzymatic activity assay. Different protein/KBr weight ratios and residual water contents were studied for their possible effects on aggregation.

Results. Depending on the pressure, a loss of enzymatic activity accompanied by an increase in soluble high molecular weight aggregates of the protein (up to ~15%) was demonstrated. Aggregation was reduced to less than 5% by a suitable dilution of the protein in KBr (1 in 1000). In contrast, water content variability (1–11 wt. %) did not affect aggregation.

Conclusions. The findings emphasize the importance to examine for protein integrity when using the KBr method for FTIR sample preparation. Protein aggregation may be minimized by optimizing the sample preparation condition such as changing the protein/KBr weight ratio.

KEY WORDS: rhDNase; FTIR spectroscopy; compression; protein aggregation; KBr pellets.

INTRODUCTION

FTIR spectroscopy has recently experienced a rapid growth in popularity in structural studies of proteins (1). Compared with other spectroscopic techniques, one particular advantage of FTIR spectroscopy is the variety of environments (liquids and solids) in which proteins can be studied. A common application of FTIR has been to investigate the secondary structural conformations of proteins in the dry state as compared to those in solution (2–5). Conformational differences demonstrated by FTIR spectroscopy may be used as criteria for the selection of excipients and protective cosolvents for proteins during lyophilization (3,4). For protein solids, the KBr pellet method is commonly used to prepare samples for FTIR spectroscopy (2–5). The preparation involves mixing the protein with KBr powder, loading the powder mix into an evacuated stainless steel die, followed by mechanical pressing of the sample to form a pellet (disc). High pressures are needed to obtain optical transparency of the KBr disc which is required to obtain high quality IR spectra. The use of high pressure, however, can cause unwanted changes in the samples. In small drug molecules, solid-state polymorphic transformations due to mechanical pressure are very common, this being observed for the non-steroidal anti-inflammatory compound phenylbutazone (6), the calcium antagonist fostedil (7) and the antibiotic succinlysufathiazole (8). In proteins, it is known that pressure can induce conformational changes of the molecules in solution (9). In the solid state, it has been observed that compactional pressure produced adverse effects on the bioactivity of some proteins (10). We recently investigated the aggregation of lyophilized recombinant human deoxyribonuclease I (rhDNase) using FTIR spectroscopy (11). In the course of the study, we found a loss in the enzymatic activity of rhDNase after the protein was compressed to form KBr pellets. The loss in activity was correlated with an increase in high molecular weight aggregates of the enzyme. These results are summarized in this report. The purpose of this note is to alert FTIR investigators to the finding that a protein may degrade when it is compressed during sample preparation using KBr pellet for FTIR spectroscopy. One must therefore verify the KBr method before using it. At present, such data are usually absent in the literature (2–5). However, interpretation of FTIR data without knowing if the proteins degrade in KBr pellets could invalidate any conclusions.

MATERIALS AND METHODS

Lyophilized powders of rhDNase were prepared from an aqueous solution of the protein at 20 mg/ml rhDNase in deionized water. The native protein in the lyophilized cake is almost entirely (99%) monomeric as ascertained by size-exclusion HPLC after reconstituting in water.

IR Sample Preparation

Effect of Compressional Pressure

Approximately 25 mg protein samples were ground and mixed with 3600 mg KBr powder (Spectra-Tech, CT), using an agate mortar and pestle. Aliquots of 200 mg of the powder mix were compressed to form discs (13 mm dia.) in an evacuated stainless steel die (Foxboro Co., MA), using 1,000–20,000 lb force, corresponding to pressures of 26 to 526 MPa (Model C, Carver Laboratory Press, IN). A triplicate run was carried out for each pressure.

Effect of Varying Protein/KBr Weight Ratio for Compression

Six different weight ratio of rhDNase to KBr were examined (1:10, 1:50, 1:150, 1:200, 1:500 and 1:1000). Approximately 600 mg of KBr were mixed with a predetermined amount of rhDNase at each of the ratios. The powder mix was divided into three equal portions, each of about 200 mg which was compressed at 526 MPa to form the KBr pellet.

Effect of Residual Water Content in the Protein

The residual water content of the lyophilized rhDNase was initially 11 ± 1% wt. Lower water contents were obtained by drying the protein (1.3 mg) on a microbalance under vacuum at 25°C to a known weight (Model MG300B, VTI Instruments). As a precaution to avoid moisture resorption, the balance was

[1] Department of Pharmaceutical Research and Development, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080.

[2] Present affiliation: Department of Pharmacy, University of Sydeny, NSW 2006, Australia.

[3] To whom correspondence should be addressed.

bleaked with dry nitrogen gas (99.99% purity) during the sample removal. The sample was then immediately mixed with 200 mg of pre-weighed KBr in a dry box and compressed at 526 MPa, both under continuous nitrogen purge. Control samples obtained under the same conditions but without compression confirmed that the drying process itself did not induce aggregation (using size exclusion HPLC).

FTIR Spectroscopy

The infrared spectra of rhDNase in the KBr discs were collected on an FTIR spectrometer (Galaxy 5022, ATI Instruments, WI) at 4cm^{-1} resolution for 300 scans. The system was continuously purged with dry air at 25 L/min (Model 75-26, FT-IR Purge Gas Generator, Balston Inc., MA). A background spectrum without the specimen was collected under the same instrumental conditions. All spectra were ratioed against the blank background.

Data Analysis

The bandwidth of amide I was measured at 85% of peak height using the region 2000–2500 cm^{-1} as the baseline. Second derivative spectra were obtained after the original spectra were smoothed with a 17-point Savitsky-Golay function (14) to remove the noise. Curve fitting were performed using the WinFirst software (14) on the Fourier self-deconvoluted spectra (15). Fourier self-deconvolution (FSD) was carried out on 9-point smoothed spectra using a full width at half-height (W) of 20 cm^{-1}, an enhancement factor (K value) of 1.5 and Bessel apodization function. To avoid artifacts due to truncation of the amide I band, the region of 1730-1550 cm^{-1} was employed for the curve fitting. Initially, six component bands were identified using both second derivative and FSD spectra (W = 30, K = 2.5) obtained from the 9-point smoothed spectra. Curve fitting was then carried out with peak centers fixed and amplitude, width and fraction Lorenzian were allowed to vary. Afterwards the peak centers were allowed to be optimized. Peak-center drift was usually minimal in this later stage of fitting. The curve-fit spectrum was superimposable on the original spectrum.

Evaluation of Changes in the rhDNase Induced by Compression

After collecting the FTIR spectra, the discs were reconstituted in 1.5 ml of deionized water (Milli-Q, Millipore Inc, CA) for bioactivity and aggregation assays. Protein aggregation was quantified by size exclusion HPLC on a 30 cm TSK 2000SWXL column (Hewlett Packard, CA). The 20 minute HPLC run was isocratic at 1.0 ml/min of mobile phase (5 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, titrated to pH 7.0). Absorbance was monitored at 280 nm. The elution times of the Bio-Rad gel filtration standards consisting of thyroglobulin (670 kD), gamma globulin (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and cyanocobalamin (1.35 kD) were used to distinguish between the rhDNase monomer and aggregates. The % aggregates was expressed as the ratio of the peak area of the aggregates to the total peak area. The bioactivity of rhDNase was assayed using an adaptation of the procedure described by Kurnick (12,13). The assay monitored the absorbance change at 620 nm resulting from the dissociation of a dye, methyl green, bound to the DNA strands. The assay diluent was 25 mM HEPES, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.1% BSA, 0.01% thimerosol, 0.05% Polysorbate 20, pH 7.55 ± 0.05. Pellet samples reconstituted with water were diluted sequentially into the diluent to final concentrations between 0.2 and 0.8 μg/ml for the assay.

RESULTS AND DISCUSSION

Effect of Compressional Pressure

Figure 1 shows the loss in methyl green enzymatic activity of rhDNase after compression. At a compression pressure of 130–260 MPa, the bioactivity decreased by about 20% and started to level off. This bioactivity change was mirrored by an increase in the aggregation of the protein (Figure 1). The size exclusion HPLC showed that the retention times for the aggregates and monomer were 5.6 and 7.6 minutes, respectively. The aggregates have a molecular size >300 kD estimated by the void volume (c.f. 33 kD for the monomer). Thus, the loss in bioactivity was largely attributed to the increase in soluble aggregates during compression (no insoluble matter was visualized in the reconstituted solutions). The KBr control sample (without compression) remained almost fully active with 98% monomeric protein, indicating that the changes observed on compression were not due to the presence of KBr in the solution. Rather, the pressure in the presence of KBr powder is responsible for the changes observed. In order to obtain transparent KBr discs, a minimum pressure of 260 MPa is needed. Below this pressure, a drift in the spectral baseline due to optical aberration becomes obvious. This is further evidenced by the observation that the amide I bandwidth measurements at lower pressures are out of line with the results at higher pressures (Figure 2), i.e., there is an initial decrease in the bandwidth at pressure below 260 MPa and an increase in the bandwidth above this pressure. However, as just described, at this pressure rhDNase will aggregate with substantial loss of its enzymatic activity. The second derivative spectra (Figure 3) show the occurrence of an amide I band component at 1634 cm^{-1} instead of 1639 cm^{-1} when the samples prepared at pressure of 260 MPa or higher. The spectral difference suggests that the aggre-

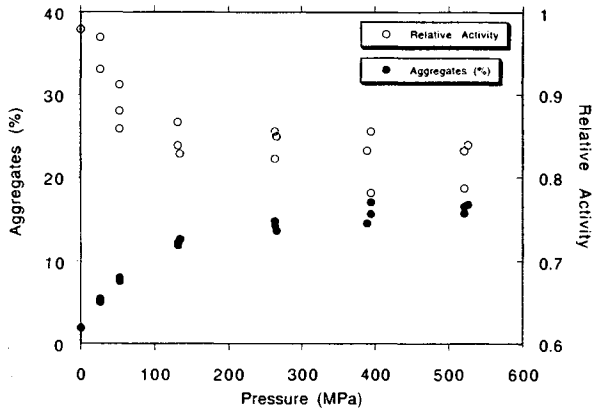

Fig. 1. The change in bioactivity and aggregation of rhDNase with compression pressure (relative activity is the rhDNase concentration measured by the methyl green assay divided by the total protein concentration measured by UV absorbance at 280 nm).

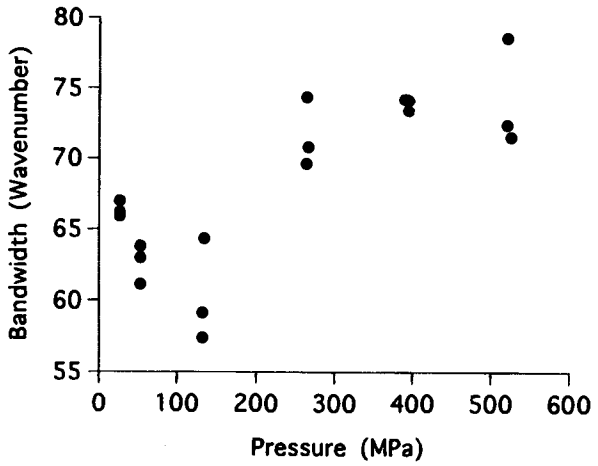

Fig. 2. The change in the amide I bandwidth with compression pressure used for preparation of the KBr pellets.

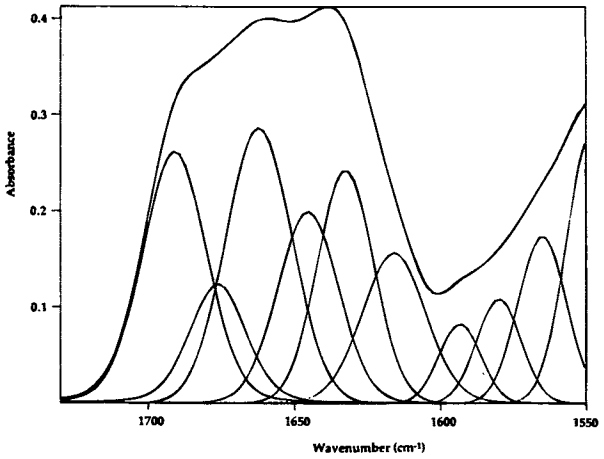

Fig. 4. Deconvoluted amide I spectrum with the best-fitted individual component bands (sample prepared at 395 MPa).

gates may involve a change of H-bonding in the β-sheet structure (1). Detailed curve fitting of the FSD spectra of rhDNase shows the amide I band contour composed of at least 6 bands at 1690, 1676, 1661, 1645, 1632 and 1616 cm^{-1} (Figure 4). The assignment of these bands can be obtained from FTIR studies on other proteins (16,17) and aggregates (18). The two bands at 1676 and 1632 cm^{-1} correspond to anti-parallel β-sheet; the band at 1661 cm^{-1} is due to turns; the bands at 1645 cm^{-1} represents the α-helix and random segments. Assignment for the bands at 1690 and 1616 cm^{-1} is less certain. The 1616 cm^{-1} band is believed to be indicative of intermolecular β-sheet structure of denatured proteins and aggregates (19–22), although C=C stretching vibration in aromatic side-chain residue and guanidine group of arginine residue have also been proposed (23,24). The 1690 cm^{-1} is assigned to intermolecular β-sheet as reported for various protein aggregates (18,25,26), but it may also be due to protonated carboxylate groups (23). If the components at 1616 and 1690 cm^{-1} represent only aggregation, a plot of the percent area of these components against pressure (Figure 5) should be close to the aggregation-pressure relationship in Figure 1. But, rather it shows a similarity to the bandwidth plot (Figure 2). Thus, the 1616 and 1690 cm^{-1} components do not completely arise from the aggregates; side-chain residues may contribute. As described earlier, spectral interference also comes from the optical aberration in samples prepared at insufficient pressures.

Effect of Protein/KBr Ratio

In order to explore the situation under which the pressure-induced aggregation could be minimized, we further studied the effect of different protein/KBr weight ratio and residual water content. In powder compression, the individual particles undergo elastic, plastic, or brittle deformation. It is well known that the pressure is not uniform, as indicated by a variation of density within a compact (27). Sharp contact points between particles will experience a higher pressure than that of the bulk, leading to the non-uniform distribution of pressure. It was proposed that the molecules at the points of contact between newly sheared surfaces of the particles are close enough so that van der Waals forces can interact to form mechanical bonding (27). For proteins, hydrophobic interactions between unfolded

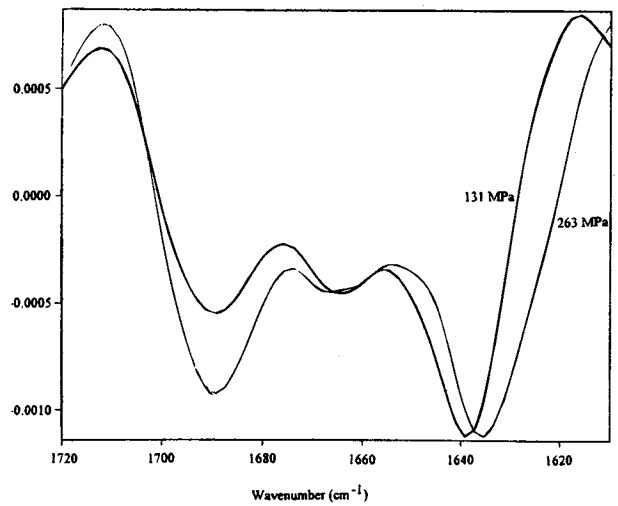

Fig. 3. Second derivative FTIR spectra of rhDNase in KBr pellets prepared at different pressures (for clarity purpose, only the spectra at 131 and 263 MPa are shown).

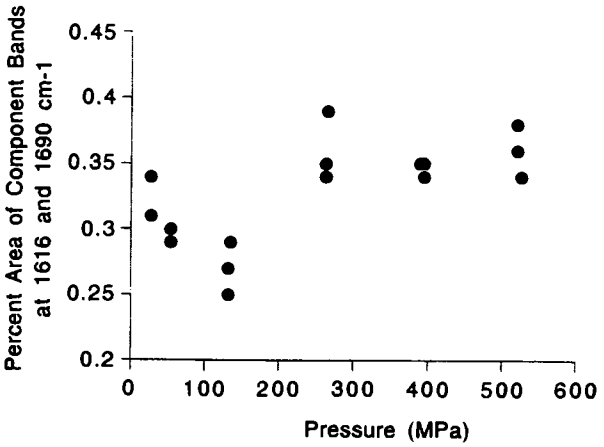

Fig. 5. Plotting the percent aggregates versus the percent area of the band components at 1616 & 1690 cm^{-1}.

molecules may also play a role for aggregation to occur. In rhDNase, less aggregation was observed as the protein/KBr weight ratio becomes smaller (Figure 6). The larger physical separation in the more diluted system may prevent direct contact between the protein particles. However, this should not affect aggregation unless it occurs preferentially at the point of particle to particle contact. Since the same bulk pressure was applied in all cases, the decreased aggregation must be due to a reduction of the *local* high pressure experienced by the protein as a result of changing the relative amount of rhDNase to KBr. Some investigators have pointed out that during powder compression heat will be generated which may contribute to degrade the protein (10,28). In practice, it is very difficult to measure the local heat change in a compact. However, heating lyophilized rhDNase under vacuum at 60 °C for 1 hr only leads to 3% aggregation (unpublished data). The results tend to support that pressure rather than heat is contributing more to aggregation of rhDNase during compression. It is suggested in Figure 6 that by extrapolation, aggregation is expected to be zero at a rhDNase/KBr weight ratio of 0.0004. As we increased the ratio from 0.001 to 0.0004 (which was a further dilution of the protein with 2.5 times of KBr), we found no more reduction in aggregation (3.6 ± 0.6 % aggregates by size exclusion HPLC, n = 3). In addition, at such a ratio the signal will be very low (~0.03 abs) and signal-to-noise ratio may become a problem.

Effect of Water Content

It is possible that the residual water content in dry proteins may affect the aggregation during compression. Since water acts as a plasticizer in an amorphous (glass) system, it will modify the mechanical deformation and pressure distribution in the protein powder. Water also affect the molecular flexibility of proteins in solid state and their thermal denaturation (29). It seems rational to expect a dependence of pressure-induced denaturation (aggregation) on water content. However, regardless of the residual water content (1, 3, 5, 8, 11 wt. %), the level of aggregation was found to be similar. The mean ranged from 18.9 to 23.0, and the standard deviation for the mean ranged from 1.7 to 5.8. Higher water contents may be impractical for FTIR measurements because of inferior optical quality of the KBr pellet and the IR absorption of water at 1640 cm^{-1} becoming significant. The lack of effect is possibly related to the BET monolayer coverage of water molecules on the protein (29,30). For instance, water content in the samples in this study might not have exceeded the monolayer. Calculation based on the number of polar sites in the rhDNase molecule gives 8.0 % (w/w) water content for the monolayer (unpublished data). However, since rhDNase is a glycoprotein with approximately 10% (w/w) carbohydrates, this value will be higher if the polar sites for carbohydrates are also included. An accurate calculation of the latter is not feasible at this stage because the glycosylation details are unknown.

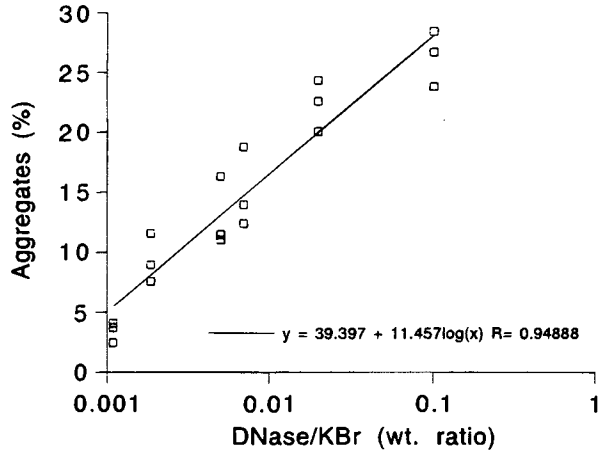

Fig. 6. The decrease in aggregation of rhDNase with protein/KBr weight ratio.

This study involved rhDNase as an example. Whether the findings are applicable to other proteins perhaps needs to be studied case by case. However, the degradation effects of compaction on the activity of other proteins such as urease, lipase, α-amylase and β-glucuronidase (10), and most recently, of catalase (28), have been documented, though not in regards to making KBr pellets. Therefore, in using the KBr pellet method for a protein an investigator has to check if the protein will degrade or not. If so, then one should first optimize the conditions for sample preparation. For example, in the present case, using a weight ratio of > 1 in 1000 for rhDNase in KBr can minimize aggregation to less than 5%. The KBr method is clearly not recommended if pressure-induced changes in the protein molecule are significant(>10%) and cannot be rectified. However, the KBr method might still be used to study *relative* changes in solid states (e.g., effect of excipient sugars in formulations), if the pressure-induced changes remain constant in all cases. In addition, techniques that do not involve the use of high pressures, such as the mull method or diffuse and internal reflectance, may be considered as suitable alternatives.

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REFERENCES

1. W. K. Surewicz, H. H. Mantsch, and D. Chapman. Determination of protein secondary structure for Fourier transform infrared spectroscopy: a critical assessment. *Biochemistry* **32**:389–394 (1993).
2. P. W. Yang, H. H. Mantsch, J. L. R. Arronda, I. Girons-Saint, G. N. Guillou, G. N. Cohen and O. Barzu. Fourier transform infrared investigation of the *Escherichia coli* Methionine Aporepressor. *Biochemistry* **26**:2706–2711 (1987).
3. J. F. Carpenter and J. H. Crowe. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* **28**:3916–3922 (1989).
4. S. J. Prestrelski, T. Arakawa, and J. F. Carpenter. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. structural studies using infrared spectroscopy. *Archives Biochem. Biophys.* **303**:465–473 (1993).
5. P. S. Belton and A. M. Gil. IR and Raman spectroscopic studies of the interaction of trehalose with hen egg white lysozyme. *Biopolymer* **34**:957–961 (1994).
6. H. G. Ibrahim, F. Pisano, and A. Bruno. Polymorphism of phenylbutazone: properties and compressional behavior of crystals. *J. Pharm. Sci.* **66**:669–673 (1977).
7. Y. Takahashi, K. Nakashima, T. Ishihara, H. Nakagawa, and I. Sugimoto. Polymorphism of fostedil: characterization and poly-

morphic change by mechanical treatments. *Drug Dev. Ind. Pharm.* **11**:1543–1563 (1985).
8. S. R. Byrn. *Solid-state Chemistry of Drugs,* Academic Press, NY, 1982, pp. 136–137.
9. J. L. Silva and G Weber. Pressure stability of proteins. *Annu. Rev. Phys. Chem.* **44**:89–113 (1993).
10. M. J. Groves and C. D. Teng. The effect of compaction and moisture on some physical and biological properties of proteins, in *Stability of Protein Pharmaceuticals Part A,* Plenum Press, New York (1992).
11. H.-K Chan and B. Ongpipattanakul. Fourier transform infrared spectroscopic measurement of thermally induced aggregates of lyophilized recombinant human DNase. *Pharm. Res.* **10**(Suppl): S79 (1993).
12. N. B. Kurnick. The determination of deoxyribonuclease activity by methyl green application to serum. *Arch. Biochem.* **29**:41–53 (1950).
13. D. C. Cipolla, A. R. Clark, H.-K. Chan, I. Gonda and S. J. Shire. Assessment of aerosol delivery systems for recombinant human deoxyribonuclease. *S.T.P. Pharma Sci.* **4**:50–62 (1994).
14. User's reference guide, WinFirst, Fourier infrared software tools from Mattson Instruments, Madison, WI (1992).
15. R. S. Jackson and P. R. Griffiths. Comparison of Fourier self-deconvolution and maximum likelihood restoration for curve-fitting. *Anal. Chem.* **63**:2557–2563 (1991).
16. D. M. Byler, H. Susi. Examination of the secondary structure of protein by deconvoluted FTIR spectra. *Biopolymers* **25**:469–487 (1986).
17. A. Dong, P. Huang, W. S. Caughey. Protein secondary structures in water from second derivative amide I infrared spectra. *Biochemistry* **29**:3303–3308 (1990).
18. A. Dong, S. J. Prestrelski, S. D. Allison and J. F. Carpenter. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. *J. Pharm. Sci.* **84**:415–424 (1995).
19. A. H. Clark, D. H. P. Saunderson and A. Suggett. Infrared and laser-raman spectroscopic studies of thermally-induced globular protein gels. *J. Pept. Protein Res.* **17**:353–364 (1981).
20. W. K. Surewicz, J. J. Leddy and H. H. Mantsch. Structure, stability, and receptor interaction of cholera toxin as studied by Fourier-transform infrared spectroscopy. *Biochemistry* **29**:8106–8111 (1990)
21. E. Labruyere, M. Mock, W. K. Surewicz et al, Structural and ligand-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase and of a catalytically inactive variant in which glutamine substitutes for lysine-346. *Biochemistry* **30**:2619–2624 (1991)
22. M. Urbanova, P Pancoska and T. A. Keiderling. Spectroscopic study of the temperature-dependent conformation of glucoamylase. *Biochim. Biophys. Acta* **1203**:290–294 (1993).
23. H. L. Casal, U. Kohler, H. H. Mantsch. Structural and conformational changes of b-lactoglobulin B: an infrared spectroscopic study of the effect of pH and temperature. *Biochim. Biophys. Acta* **957**:11–20 (1988).
24. Y. N. Chirgadze, O. W. Fedorow and N. P. Trushina. Estimation of amino acid residue side-chain absorption in the infrared spectra of protein solutions in heavy water. *Biopolymers* **14**:679–694 (1975)
25. D. Naumann, C. Schultz, U. Gorne-Tschelnokow and F. Hucho. Secondary structure and temperature behavior of the acetycholine receptor by Fourier transform infrared spectroscopy. *Biochemistry* **32**:3162–3168 (1993).
26. H. Fabian, C. Schultz, D. Naumann, O. Landt, U. Hahn and W. Saenger. Secondary structure and temperature-induced unfolding and refolding of ribonuclease T1 in aqueous solution, a Fourier transform infrared spectroscopic study. *J. Mol. Biol.* **232**:967–981 (1993).
27. E. L. Parrott, Compression. In H. A. Lieberman and L Lachman (eds.), *Pharmaceutical Dosage Forms: Tablets Volume 2,* Marcel Dekker, Inc., New York, 1981, pp. 153–184.
28. D. E. Wurster and R. L. Ternik. Pressure-induced activity loss in solid state catalase. *J. Pharm. Sci.* **84**:190–194 (1995).
29. M. J. Hageman. Water sorption and solid-state stability of proteins, in *Stability of Protein Pharmaceuticals Part A,* Plenum Press, New York (1992).
30. C. C. Hsu, C. A. Ward, R. Pearlman, H. M. Nguyen, D. A. Yeung and J. G. Curley. Determining the optimal residual moisture in lyophilized protein pharmaceuticals. *Develop. Biol. Standard* **74**:255–271 (1991).