Second Derivative Infrared Spectroscopy as a Non-Destructive Tool to Assess the Purity and Structural Integrity of Proteins

D. Michael Byler,[1,3] Rachel M. Wilson,[1] Cynthia S. Randall,[2] and Theodore D. Sokoloski[2]

Received July 7, 1994; accepted October 12, 1994

Second derivative infrared (IR) spectroscopy can be used as a quick, easy, reproducible, cost-effective, non-destructive tool by which to evaluate the purity and structural integrity of samples of water-soluble proteins from a variety of sources. For this study, second derivative IR spectra were collected at ambient conditions for aqueous (D_2O) solutions of seven different commercial samples of the same enzyme, porcine pancreatic elastase (2.0 to 3.8 mg protein/100 μL D_2O, pD = 5.4 to 9.1). As with other globular proteins possessing a large fraction of β-structure, the amide I′ region [1700-1620 cm^{-1}] of the second derivative IR spectra for each of the seven elastase samples exhibits a characteristic pair of bands: one of weak intensity appears near 1684 cm^{-1}; the other close to 1633 cm^{-1} is moderate-to-strong. However, one of the seven samples shows a striking decrease in the observed intensities of the amide I′ bands relative to the 1516 cm^{-1} absorption, along with the appearance of a strong, new band at 1614 cm^{-1}. These intensity disparities strongly suggest that this sample is of much lower quality than the others and clearly has an appreciable proportion of the protein present in a non-native state. In addition, minor differences evident in the position and relative intensity of some individual amide I′ bands among the seven spectra imply that subtle variations exist in the conformation of the peptide backbone of the seven samples. For two of the samples, these small, but reproducible, changes seem to be correlated with marked losses of enzyme activity. Finally, bands outside the amide I′ region may prove useful in assessing sample purity and identifying non-protein contaminants.

KEY WORDS: elastase; protein conformation; protein structure; protein purity; infrared (IR); second derivative spectra; SDS-PAGE electrophoresis; enzyme activity.

INTRODUCTION

Proteins, enzymes, and peptides are becoming increasingly important as potential pharmaceuticals. As such, clear criteria for evaluating their purity and structural integrity have become all the more critical. Of particular interest is the development of reliable, quick, easy, reproducible, cost-effective, non-destructive methods by which one can assess these factors for samples of water-soluble proteins and enzymes from a wide variety of sources. Fourier-transform infrared (IR) spectroscopy (1-4) offers many advantages which can complement other traditional methods of analysis, including gel electrophoresis and measurement of enzyme activity (5). IR is particularly sensitive and useful when coupled with second derivative, Fourier deconvolution, or difference techniques (1-4,6,7) to search for small changes in the structure of a specific protein. Nor is resolution-enhanced IR spectroscopy restricted to the examination of proteins in solution, for this technique can be used to probe the conformation of solid samples as well (8).

The widely studied and well characterized enzyme, porcine pancreatic elastase [EC #3.4.21.11], was selected as a representative protein for examination in this investigation (9,10). It is available from a variety of commercial sources, in different degrees of activity and purity. Elastase is a serine protease consisting of a single, 25900 dalton peptide chain; four disulfide bridges stabilize its three-dimensional structure. According to one widely referenced analysis of the X-ray crystal structure (11), the backbone conformation of this protein consists of 47% β-structure, 10% α-helix, as well as turns and other less periodic structures. Estimates made by iterative curve fitting of the amide I′ band components [1700-1620 cm^{-1}] of the Fourier deconvolved infrared spectrum gave similar values (12); amide I′ absorptions arise principally from the C=O stretching vibrations of the peptide backbone.

The results of this study showed that some of the seven elastase samples analyzed were of rather low purity and that some had been denatured to a greater or lesser extent. Correlations between variations in the observed spectra and apparent differences in the purity, extent of denaturation, and loss of activity of the enzyme sample are discussed.

MATERIALS AND METHODS

Seven different samples of porcine pancreatic elastase (MW = 25900 daltons) were obtained from the following four sources: Sigma Chemical Co., St. Louis, MO (Cat. #E-1027, type III, chromatographically purified, lyophilized); Calbiochem Corp., La Jolla, CA (Cat. #324689, dialyzed, lyophilized, contains sodium acetate); Worthington Biochemical Corp., Freehold, NJ (two different types: Cat. #6365, code #ESFF, 95% protein, chromatographically prepared, lyophilized and Cat. #2292, code #ESL, 93% protein, 2× crystallized, lyophilized); and Fluka Biochemika, (Cat. #45125, lyophilized, salt-free). D_2O (99.9% D) was from Aldrich Chemical Co., Milwaukee, WI. All samples, except the one from Fluka were colorless, white powders and dissolved completely to give colorless, transparent solutions. The Fluka sample was a very faint yellow and gave a pale yellow solution, with traces of insoluble matter. All samples had been stored at *ca.* −20°C for varying lengths of time.

Because we do not wish to recommend one company's products as better than another's, in the discussion which follows, we have chosen to denote the seven enzyme samples **randomly** and arbitrarily as **A, B, C_1, C_2, D_1, D_2,** and **E**. For example, sample A represents one of the seven samples, but not necessarily the one from Sigma. Samples C_1 and C_2 represent two different lots of a single type of elastase from the same source; likewise, so do samples D_1 and D_2.

[1] Department of Chemistry and Physical Science, Philadelphia College of Textiles and Science, Philadelphia, Pennsylvania 19144-5497.

[2] Department of Pharmaceutical Technologies, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939.

[3] To whom correspondence should be addressed.

0724-8741/95/0300-0446$07.50/0

Samples for infrared spectroscopic analysis were prepared by dissolving 2.0 to 3.8 mg of the lyophilized enzyme in 100 μL D_2O with no added buffer or salts. The pD's of the resulting solutions encompassed a range of values, but were not adjusted; uncorrected pD values were measured with a semi-micro combination pH electrode and a Corning digital pH meter calibrated with pH 7.00 and pH 4.00 buffers (Baxter Diagnostics, Inc., Deerfield, IL) at a constant room temperature (*ca.* 23 ± 1°C). The respective concentrations (%, w/v) and pD's for the seven samples were **A**: 2.4%, pD = 5.7; **B**: 3.8%, pD = 8.7; C_1: 2.2%, pD = 7.3; C_2: 2.1%, pD = 7.2; D_1: 4.0%, pD = 9.1; D_2: 2.0%, pD = 8.0; **E**: 2.0%, pD = 5.4.

The demountable Specac IR cell had CaF_2 windows and a nominal 50 μm lead spacer. This cell has a volume of less than 25 μL.

IR spectra were obtained with a Nicolet 510 spectrometer equipped with a air-cooled Globar source, Ge-coated KBr beam splitter, a room-temperature DTGS detector, an 18-bit analog-to-digital converter, and a Nicolet 620 workstation with NICOS operating system and DX FTIR software. All spectra were collected at a nominal resolution of 2 cm^{-1} by co-adding 1024 single-sided interferograms and then Fourier transforming the data after application of a Happ-Genzel apodization function. The 10240 data points of each interferogram are in single precision, except for the 512 points surrounding the centerburst. The latter are double precision. The mirror velocity was 0.76 scans/s (VEL = 30). The spectrometer and its sample chamber were continuously purged with dry nitrogen gas. Second derivative spectra were obtained by applying program DERIV twice in succession to the original absorbance data. No smoothing function was applied. Residual water vapor lines were eliminated from the spectra as previously described (12).

To eliminate absorption due to the very broad, but weak, combination band of the D_2O solvent near 1555 cm^{-1} (13), the spectrum of the solvent was subtracted interactively with the subtraction factor chosen such that another D_2O combination band near 3843 cm^{-1} was effectively removed, leaving a flat baseline. H-D exchange between the D_2O solvent molecules and the protons of the peptide NH groups and other exchangeable hydrogens of the amino acid side chains results in the formation of small amounts of HOD. This molecule has fundamental vibrations near 3408 and 1461 cm^{-1}. Careful interactive subtraction of an HOD spectrum eliminated these features to give a zero baseline near 3408 cm^{-1}. The requisite HOD spectrum was obtained by subtracting the spectrum of the nearly pure D_2O (99.9% D) from a spectrum of D_2O containing approximately 1% H_2O (v/v).

The peak absorptions of all spectra, both the original absorption data and the second derivative spectra, were normalized by multiplying every ordinate value of each spectrum by a factor calculated to give a constant value for the second derivative peak intensity of the tyrosine ring stretching mode at 1516 cm^{-1} (4,6,14). This frequency is 2-3 cm^{-1} higher than the measurements reported by Bendit (14). His values, however, are for solid cast films of poly-tyrosine and representative proteins, while those given here are for aqueous solutions. This tyrosine band was chosen as the intensity benchmark because it is rather isolated from other absorptions and is sharp and easily discernible, especially in the second derivative spectra. Furthermore, the frequency of this band is normally virtually unaffected by a wide range of environmental perturbations. Deuteration of the hydroxyl hydrogen results in a small, constant downward shift of about 1 cm^{-1} (from 1517 to 1516 cm^{-1}) and, according to Bendit (14), causes no appreciable change in band intensity. Only when the hydroxyl group becomes ionized at relatively high pH (>10 or so) or when the hydroxyl is strongly hydrogen bonded does the frequency undergo greater change; then typical decreases are 5-7 cm^{-1} (6). Thus, the position and intensity of this band are essentially invariant even if the conformation of the protein changes or if the protein is cut into smaller peptides by proteolysis. Indeed, Bendit (14) notes that the integrated 1516 cm^{-1} band area is proportional to the number of tyrosines present in the protein or polypeptide sample.

SDS polyacrylamide gel electrophoresis of the seven protein samples was carried out as described by Laemmli (15) under reducing conditions using 0.2 M dithiothreitol. Gels were stained with Coomassie blue and mobilities of the polypeptides were calculated relative to the mobilities of six standard proteins (Integrated Separation Systems, Natick, MA): phosphorylase b (95,000 daltons); bovine serum albumin (68,000 daltons); alcohol dehydrogenase (39,000 daltons); carbonic anhydrase (29,000 daltons); trypsin inhibitor (20,400 daltons); and lysozyme (14,000 daltons).

Elastase activities were determined using N-succinyl-(L-alanyl)$_3$-*p*-nitroanilide (Calbiochem Corp., La Jolla, CA) as substrate, according to a method (16) modified from Feinstein *et al.* (17).

Protein assays for each sample were carried out by standard dye binding methods (18) using Coomassie Blue G-250 (Coomassie Protein Assay Reagent, Cat. #23200 G, Pierce, Rockford, IL).

RESULTS

Infrared Absorption Spectra of Elastase

Seven elastase samples obtained from commercial sources were analyzed by infrared spectroscopy. As is characteristic of proteins having a large fraction of β-structure 1–4,12), the amide I′ region of the second derivative spectra of the seven samples in D_2O solution all exhibit a distinctive pair of bands near 1684 [weak] and 1633 cm^{-1} [moderate-to-strong] (Fig. 1). As many as five or six other relatively weak bands due to other types of secondary structure components may be clearly observed as well. With the exception of sample A, the IR spectra in the amide I′ region are superficially similar: the 1633 cm^{-1} second derivative band displays a relative peak intensity greater than or equal to that of the normalized peak height of the tyrosine phenyl ring stretching mode at 1516 cm^{-1}. For sample A, however, the 1633 cm^{-1} amide I′ band is significantly weaker than the tyrosine absorption.

The spectrum of sample A is also distinctive because of the unusually high intensity of a band at 1614 cm^{-1}. Indeed, for sample A, this band is much stronger than the band at 1633 cm^{-1} (Fig. 1). Of the other samples, only samples D_1 and D_2 exhibit appreciable absorption at this frequency.

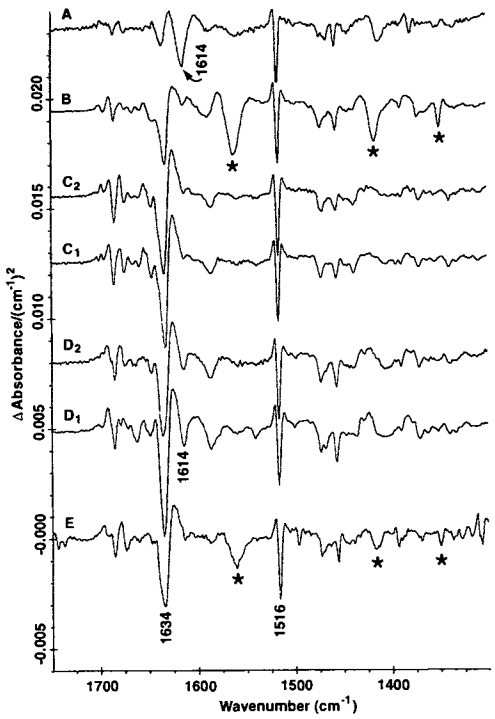

Fig. 1. Normalized second derivative infrared spectra of seven elastase samples. The peaks in spectrum B and spectrum E marked with an asterisk are due to the presence of acetate ions.

Even the ordinary, undifferentiated IR absorption spectrum of sample A is unique (Fig. 2): instead of having a single, broad, strong absorption near 1635 cm^{-1} as do the other elastase samples, the amide I′ peak of sample A appears only as an incompletely resolved shoulder on a much stronger band at 1614 cm^{-1}.

The unique character of the amide I′ IR spectrum of this sample is unrelated to the pD (5.7) observed for this solution. Sample E has an even lower pD value (5.4), but its amide I′ spectrum it more nearly like those for samples B, C_1, and C_2, whose pD's equal 8.7, 7.3, and 7.2, respectively.

In the region below 1500 cm^{-1}, the second derivative spectra of all seven samples have in common a number of distinct, relatively sharp bands. The most important appear around 1585, 1516, 1472, 1457, 1438 cm^{-1} (Fig. 1). Spectra B and E have additional rather strong absorptions near 1561, 1416, and 1350 cm^{-1} which are not found in the spectra of the other five samples (Figs. 1, 2). Sample E alone among the seven samples also has a strong band at 1101 cm^{-1} (not shown).

Gel Electrophoresis and Enzyme Activity Measurements of Elastase

Subsequent to the infrared examination, the seven elastase samples were analyzed by SDS polyacrylamide gel electrophoresis (15). At normal loadings, each sample consisted of just one polypeptide with a relative mobility of 26,000 daltons, except for sample A which exhibited a second polypeptide of comparable intensity with a relative mobility of 32,000 daltons. The other samples, however, did display traces of a 32,000 dalton band when the gel was overloaded.

Measured elastase activities, in units of $(\Delta A_{410}/min)/(mg\ protein/mL)$, using N-succinyl-(L-alanyl)$_3$-*p*-nitroanilide as substrate (16,17), varied widely for the seven samples: A: 1.9 units; B: 4.8 units; C_1: 5.4 units; C_2: 5.1 units; D_1: 0.3 units; D_2: 1.9 units; E: 3.1 units.

The purity of the samples in mass percent as estimated by Coomassie Blue dye binding measurements was diverse also: A: 50%; B: 57%; C_1: 87%; C_2: 93%; D_1: 41%; D_2: 32%; E: 36%.

These analytical results clearly show the extent to which the seven elastase samples investigated here differ from one another with respect to their structural integrity and purity. In particular, only samples C_1 and C_2 are both free of significant amounts of other proteins or peptides and exist in a native state with a high level of activity.

DISCUSSION

Second derivative IR spectroscopy immediately discloses that sample A is of questionable quality. In contrast to the spectra for the other six samples, the amide I′ band at 1633 cm^{-1}, generally associated with the β-strands of glob-

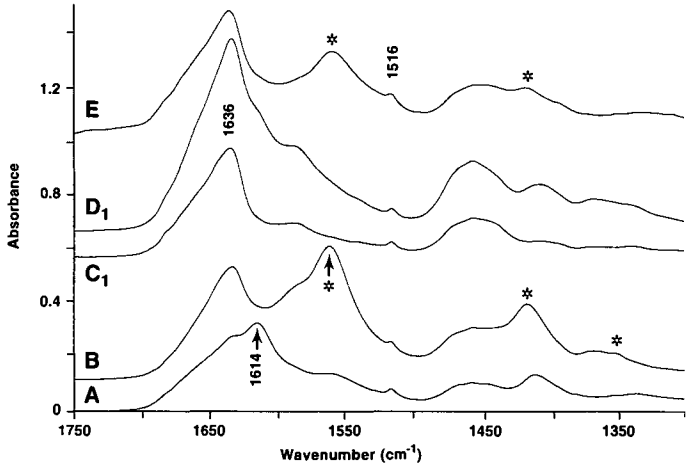

Fig. 2. Normalized absorption infrared of five elastase samples. The peaks in spectrum B and spectrum E marked with an asterisk are due to the presence of acetate ions. (C_2 and D_2 give spectra essentially identical to those of C_1 and D_1, respectively.)

ular proteins (1–4,6), is much weaker than that of the 1516 cm^{-1} mode of the tyrosine side chains. This anomaly may have occurred in one of several ways: one, this specimen may be contaminated with a large quantity of a peptide or protein composed of a much greater fraction of tyrosine than elastase. Two, this sample might have undergone a drastic loss in the number of peptide carbonyls. Presumably this might occur if the peptide backbone of a large fraction of the macromolecules in the samples were cut in numerous places, as could happen if traces of an active proteolytic enzyme were present. Three, the average molar absorptivity of the amide I′ bands of sample A may be lower than for the other samples. (The molar absorptivity of the 1516 cm^{-1} tyrosine band (14) is not likely to have changed.) Or, four, the amide I′ bands may have so broadened due to increased disorder in the protein secondary structure that the intensity of the resulting second derivative bands may have been greatly diminished. Second derivative band intensities are directly proportional to the original peak intensity but inversely proportional to the square of the original band width (6).

For the first hypothesis to be true, the contaminating protein must have 3 or 4 times as many tyrosines as elastase. This seems improbable. The electrophoresis data cast doubt upon explanation two. Sample A consists of a mixture of two polypeptides, one with the same molecular weight as the native protein and one which is perhaps an aggregate. In addition, lower molecular weight fragments indicative of possible proteolysis are absent at normal protein loadings. The third conjecture is also unlikely as no one has reported any marked difference (even qualitatively) in the molar absorptivity of the amide I′ band envelope for typical proteins. Consequently, the last supposition seems most plausible. Thus, the unusual appearance of the amide I′ bands in the second derivative spectrum of sample A probably results from increased structural disorder in some fraction of the protein molecules and the concomitant broadening of the corresponding amide I′ band components. Furthermore, the original IR absorption spectra for all of the samples exhibit amide I′ band envelopes with similar peak intensities after correction for variation in concentration and cell pathlength.

The other striking feature of the spectrum of sample A is the presence of a very strong band at 1614 cm^{-1}. Such a band often appears in the spectra of proteins and polypeptides which have been subjected to denaturation or aggregation by exposure to a non-aqueous solvent, a strongly alkaline environment, or high temperature (19–21) and is considered indicative of the formation of strong *inter*molecular hydrogen bonds between peptide linkages of β-strands on two different protein molecules. Clearly, these two factors (the low amide I′ intensity and the intense absorption at 1614 cm^{-1}) suggest that much of elastase sample A is no longer in its native state.

Differences among the seven samples in the observed extent of aggregation (intensity of the 1614 cm^{-1} band) was not due to differences in the length of time that the enzyme was in solution. All spectra were taken within about 30-40 minutes of the time of mixing with D_2O. Furthermore, additional spectra of sample C_1, among others, taken as long as 12-24 hours after dissolution showed no evidence of increased aggregation. (Nor do these spectra suggest any significant increase in the extent of deuterium exchange after about the first half hour following dissolution of the protein sample in D_2O.)

A second derivative infrared spectrum of solid, lyophilized sample A (not shown) gives no evidence of aggregation in the protein as supplied by the manufacturer. (That is, no intense second derivative band appears near 1615 cm^{-1}) Nonetheless, the amide I region of this sample is distinctly different from that of an analogous spectrum of a second solid sample of the enzyme known to be in the active native state (sample C_1). Clearly, this suggests that sample A already existed with an altered backbone conformation in the solid state prior to dissolution in water and that probably this structural alteration made this enzyme sample susceptible to rapid aggregation in aqueous solution.

Gel electrophoresis confirmed the unique character of sample A. Alone among the seven samples, this specimen consisted of two polypeptides in roughly equal amounts; one with the same relative mobility (26,000 daltons) as all the other six enzyme samples and the other with a relative mobility of 32,000 daltons. The latter presumably is some kind of denatured aggregate. Sample A also exhibited less than half the activity measured for the most active samples (B, C_1, and C_2).

Detailed comparison of the weaker amide I′ bands of the second derivative spectra (Fig. 1) of the other six enzyme samples clearly reveals small, but reproducible, variations in the position and relative intensity of these bands. These IR results strongly suggest that the various samples, while similar in the average fraction of α-helix and β-structure present, are not identical in every conformational detail (1–3). The wide variation in measured enzyme activities confirms this supposition. Samples D_1 and D_2 exhibit especially low activities. Perhaps the distinct, small, increase in the intensity of the 1661 cm^{-1} band observable for sample D_1 and no others (Fig. 1) is associated with a minor, but critical, alteration of the conformation at the enzyme's active site. Many workers have attributed bands near this frequency to some kind of "turn" (2–4). Further work will be necessary to verify such a hypothesis, however.

Notably, these two samples were the only ones besides samples A to have appreciable absorption at 1614 cm^{-1}. Yet, electrophoresis measurements gave no apparent indication of a second polypeptide as observed for sample A.

Other minor differences noted in the amide I′ bands for some of the samples may result from small structural perturbation induced by variations in pD of the solutions. However, additional investigations will be required to confirm these observations.

For a pure protein or enzyme, infrared absorption results not only from the vibrations of the peptide linkages (the so-called amide bands), but also from the normal modes of the amino acid side chains. With the exception of those groups which are ionizable or participate in the formation of strong hydrogen bonds, the observed infrared absorption frequencies associated with any given amino acid group are likely to be largely invariant from sample to sample, or even from one protein to another (14). For most proteins, no infrared bands are found between 2200 and 1700 cm^{-1}. Thus, this region serves to verify that the signal-to-noise ratio for a given spectrum is sufficiently high and that water vapor ab-

sorption lines have been effectively eliminated (1,4,12). Both these criteria must be established to ensure that results obtained by differentiation, deconvolution, or subtraction of the spectroscopic data are reliable. As for the region below 1620 cm^{-1}, bands observed there are generally rather weak. Thus, even in the second derivative spectra, where band narrowing allows individual peaks to be more easily discerned, the number of non-amide bands visible is relatively small.

The small number of such bands in the second derivative spectra for samples C_1 and C_2 (Fig. 1) implies that these samples are largely pure protein, free from contaminants. Coomassie Blue dye binding measurements confirm this observation. On the other hand, the strong bands seen at 1562, 1416, and 1349 cm^{-1} in the second derivative spectrum (Fig. 1) and the undifferentiated spectrum (Fig. 2) of sample B indicate conclusively that this specimen is much less pure. In fact, the observed frequencies of these three bands strongly suggest that sample B contains a substantial quantity of a simple carboxylate salt (probably an acetate buffer) (22). This suspicion was later confirmed by the company that supplied this sample. Similar bands are also seen for sample E (Fig. 1), although this sample also has many additional weak bands not found in a purer sample (such as C_1) due to the presence of other kinds of non-proteinaceous material.

The second derivative spectrum of sample E (Fig. 1) has two very weak peaks at 1743 and 1737 cm^{-1}. These are probably associated with the carbonyl (C=O) stretching vibration of esters, perhaps due to traces of lipids or other carboxylic acid derivatives (23,24). In addition, a very intense absorption at 1101 cm^{-1} (not shown) may well be due to the presence of simple inorganic sulfate salts (23).

CONCLUSION

Clearly, second derivative infrared spectroscopy offers an alternate means by which to assess the purity and structural integrity of many types of water-soluble proteins. This method is relatively quick and easy, economical, nondestructive, and reproducible. Less than one hour is required for each sample, including solution preparation, data collection, and analysis. Generally, less than one mg of protein dissolved in 50 μL of D_2O is sufficient. This infrared technique can complement traditional analyses of protein homogeneity by SDS gel electrophoresis: IR spectra can detect the presence of non-peptide materials and can indicate whether the enzyme is in its native conformation. IR data also complements the usual assays of enzyme activity which cannot easily distinguish between a given quantity of impure, but highly active, material and the same quantity of pure, but partially inactivated, enzyme. In addition, IR can serve to assay proteins with no enzymatic properties. With further development, infrared spectroscopy has the potential to become a valuable new tool for the working biochemist and bioengineer to evaluate the quality not only of enzymes, but also of inactive proenzymes, as well as other non-enzymatic proteins and polypeptides.

REFERENCES

1. W. Surewicz, H. H. Mantsch, and D. Chapman. Determination of protein secondary structure by Fourier transform infrared spectroscopy: A critical assessment. *Biochemistry* 32:389-394 (1993).
2. J. Bandekar. Amide modes and protein conformation. *Biochim. Biophys. Acta* **1120**:123-143 (1992).
3. W. Surewicz and H. H. Mantsch. New insight into protein secondary structure from resolution-enhanced infrared spectra. *Biochim. Biophys. Acta* **942**:115-130 (1988).
4. H. Susi and D. M. Byler. Resolution-enhanced Fourier transform infrared spectroscopy of enzymes. *Meth. Enzymol.* **130**:290-311 (1986).
5. R. K. Scopes. *Protein Purification: Principles and Practice.* Springer-Verlag, New York, 1982, pp. 213-259
6. H. Susi and D. M. Byler. Protein structure by Fourier transform infrared spectroscopy: Second derivative spectra. *Biophys. Biochem. Res. Commun.* **115**:391-397 (1983).
7. R. J. Markovich and C. Pidgeon. Introduction to Fourier transform infrared spectroscopy and applications in the pharmaceutical sciences. *Pharm. Res.* 8:663-675 (1991).
8. 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. *Arch. Biochem. Biophys.* **303**:465-473 (1994).
9. J. G. Bieth. Elastases: Catalytic and biological properties. In R. P. Mecham (ed.), *Biology of Extracellular Matrix: Regulation of Matrix Accumulation,* Academic Press, New York, 1986, pp. 217-320.
10. W. Bode, E. Meyer, Jr., and J. C. Powers. Human leukocyte and porcine pancreatic elastase: X-ray crystal structures, mechanism, substrate specificity, and mechanism-based inhibitors. *Biochemistry* 28:1951-1963 (1989).
11. M. Levitt and J. Greer. Automatic identification of secondary structure of globular proteins. *J. Mol. Biol.* **114**:181-239 (1977).
12. S. J. Prestrelski, D. M. Byler, and M. N. Liebman. Generation of a substructure library for the description and classification of protein secondary structure. II. Application to spectra-structure correlations in Fourier transform infrared spectroscopy. *Proteins: Struct. Func. Gen.* **14**:440-450 (1992).
13. G. Zuber, S. J. Prestrelski, and K. Benedek. Application of Fourier transform infrared spectroscopy to studies of aqueous protein solutions. *Anal. Biochem.* **207**:150-156 (1992).
14. E. G. Bendit. Infrared absorption of tyrosine side chains in proteins. *Biopolymers* 5:525-533 (1967).
15. U. K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**:680-687 (1970).
16. Worthington, C. C., ed. Worthington Biochemical Manual. Worthington Biochemical Corp., Freehold, NJ, 1988. p. 141.
17. G. Feinstein, A. Kupfer, and M. Sokolovsky. *N*-Acetyl-(L-ala)$_3$-*p*-nitroanilide as a new chromogenic substrate for elastase. *Biochem. Biophys. Res. Commun.* **50**:1020 (1973).
18. M. M. Bradford. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
19. M. Jackson and H. H. Mantsch. Halogenated alcohols as solvents for proteins: FTIR spectroscopic studies. *Biochim. Biophys. Acta* **1118**:139-143 (1991).
20. D. Carrier, H. H. Mantsch, and P. T. T. Wong. Pressure-induced reversible changes in secondary structure of poly(L-lysine): An IR spectroscopic study. *Biopolymers* 29:837-844 (1990).
21. D. M. Byler and J. M. Purcell. FTIR examination of thermal denaturation and gel-formation in whey proteins. *Proc. Soc. Photo Opt. Instrum. Eng. (Fourier Transform Spectroscopy)* **1145**:415-417 (1989).
22. K. Ito and H. J. Bernstein. Vibrational spectra of the formate, acetate, and oxalate ions. *Can. J. Chem.* 34:170-178 (1956).
23. N. B. Colthup, L. H. Daly, and S. E. Wiberley. *Introduction to Infrared and Raman Spectroscopy,* Academic Press, Boston, 3rd ed., 1990.
24. D. C. Lee and D. Chapman. Infrared spectroscopic studies of biomembranes and model membranes. *Biosci. Rept.*, 6:235-256 (1986).