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Use of Infrared Spectroscopy to Assess Secondary Structure of Human Growth Hormone within Biodegradable Microspheres

TZUNG-HORNG YANG,[†] AICHUN DONG,[‡] JEFF MEYER,[†] OLUFUNMI L. JOHNSON,[§] JEFFREY L. CLELAND,[||] AND JOHN F. CARPENTER*,[†]

Contribution from *School of Pharmacy, Box C238, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Denver, Colorado 80262, Department of Chemistry and Biochemisty, University of Northern Colorado, Greeley, Colorado 80639, Formulations Department, Alkermes, Cambridge, Massachusetts 02139, and Pharmaceutical R & D, Genentech, S. San Francisco, California 94080.*

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Abstract □ The purpose of this study was to test the utility of infrared (IR) spectroscopy to determine protein secondary structure in biodegradable microspheres. Encapsulation of proteins within biodegradable polymers, [e.g. poly(lactic-*co*-glycolic acid) (PLGA)] for controlled drug release has recently been the subject of intense research effort. The ability to assess protein integrity after microsphere production is necessary to successfully produce microspheres that release native proteins. We used IR spectroscopy, a noninvasive method—as opposed to conventional organic solvent extraction or in vitro release at elevated temperature—to assess the secondary structure of recombinant human growth hormone (rhGH) within dry and rehydrated microspheres. PLGA microspheres containing rhGH with different excipients were prepared by a conventional double-emulsion method. The protein IR spectra indicated that the encapsulation process could perturb the structure of rhGH and that excipients could inhibit this damage to varying degrees. A strong positive correlation was found between intensity of the dominant α-helical band in the spectra of rhGH in rehydrated microspheres and the percent monomer released from microspheres during incubation in buffer. We also studied microspheres prepared with zinc-precipitated rhGH. The addition of Zn^{2+} during microsphere processing partially inhibited protein unfolding and fostered complete refolding of rhGH upon rehydration. In conclusion, IR spectroscopy can serve as a valuable tool to assess protein structure within both dried and rehydrated microspheres.

Introduction

Over the past several years, there have been numerous reports of encapsulation of proteins within biodegradable microspheres (for a recent review, see ref 1), which are used for sustained release of therapeutic proteins. For these systems to be successful clinically it is essential that the encapsulated protein be released in a native, functional state. In addition, the structure of the protein in the dried microsphere product could greatly affect the storage stability of the encapsulated protein. However, the integrity of the encapsulated protein is difficult to assess. Organic solvents used in the fabrication process may be required to remove the protein from the microspheres for characterization, and this extraction process may itself cause degradation of the protein. Alternatively, the microspheres may be incubated in aqueous buffers at elevated temperatures (e.g., 37 °C) to facilitate erosion of the polymer matrix and release of the protein into the buffer. During this incubation period, the protein may undergo both physical and chemical degradation. Thus, protein degradation caused by the method of removing the protein from

* Author to whom correspondence should be sent. Telephone: 303-315-6075. Fax: 303-315-6281. E-mail: john.carpenter@UCHSC.edu.
[†] School of Pharmacy.
[‡] Department of Chemistry and Biochemistry.
[§] Formulations Department.
[||] Pharmaceutical R & D.

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the microspheres could prevent the accurate determination of protein integrity within microspheres.

Such assessment is necessary to ensure production of microspheres that release native proteins. For example, with a valid method, excipients may be screened for their ability to stabilize the protein during encapsulation and drying, and during rehydration under physiological conditions. The most valuable method is one that allows the encapsulated protein to be studied directly in the microspheres, without the need for extraction or dissolution steps. Infrared (IR) spectroscopy is one promising method because it can be used to study directly the secondary structure of proteins in any state, including dried solids, precipitates and, potentially, intact microspheres.[2−5] In this study, we investigated the utility of this method to determine the structure of recombinant human growth hormone (rhGH) in polylactic-*co*-glycolic acid (PLGA) microspheres. The secondary structure of rhGH in both dry and rehydrated, intact microspheres was compared with that for the native, aqueous soluble protein. Several different formulations of rhGH were screened for their ability to maintain the protein in its native state during a double-emulsion encapsulation process.[6] The degree of nativelike structure was compared with recoveries of native protein with the traditional in vitro release and organic solvent extraction methods.[7−9] Finally, the structure of rhGH was assessed in microspheres prepared by an alternative strategy, which employed protein that was precipitated with Zn^{2+} and spray-freeze-dried prior to encapsulation in PLGA.[7]

Experimental Section

Microsphere Preparation—Two different encapsulation processes were used to produce PLGA microspheres containing rhGH. PLGA polymer (RG502, RG752, and RG756) was purchased from Boehringer Ingelheim, and rhGH was supplied by Genentech Production Recovery Operations. The first encapsulation method was the conventional water-in-oil-in-water double emulsion process, which was performed as described previously.[6] To compare the results of secondary structural analysis obtained with IR spectroscopy to results obtained with dissolution and extraction procedures, microspheres prepared with rhGH containing various different excipients (e.g., mannitol, trehalose, carboxymethylcellulose, and dextran) were studied.[6] These different formulations had already been shown to provide a range of protein stabilities, as assessed by extraction of the native protein from the microspheres.[6] The same samples of these formulations were used here to determine if there would be a corresponding range in the degree of native secondary structure retention in the encapsulated rhGH.

With the second encapsulation method, rhGH was initially precipitated with Zn^{2+} and then spray-freeze-dried.[7] The protein powder was homogenized in methylene chloride containing PLGA and sprayed into liquid nitrogen and solid ethanol, as discussed previously.[7] For both methods, the final microspheres were stored as a dry powders at 2−8 °C prior to analysis.

Analysis of rhGH Release and Extraction from Microspheres Produced by the Double-Emulsion Procedure—To quantify the in vitro release of rhGH from PLGA, the microspheres were incubated in isotonic HEPES buffer, pH 7.4, at 37 °C for 1 h, using a previously described method.[8,9] The protein was extracted from a parallel set of microspheres by dissolution in methylene chloride, as previously described.[7] Protein released or extracted was analyzed by native size exclusion chromatography (SEC) to determine the amount of native monomeric rhGH.[8]

Analysis of Infrared Spectra—The IR spectra were obtained with a Nicolet Magna 550 spectrometer equipped with a DTGS KBr detector. For dry powder and microspheres samples, 0.5−1 mg of sample was mixed with 300 mg of KBr and annealed into disks. This process has previously been shown not to alter the IR spectra of dried proteins.[4] The aqueous protein or rehydrated microspheres samples were placed in a variable path length sample cell containing CaF_2 windows, which were separated by ca. 6 μm.

For each spectrum, 64 or 256 scans for dry or liquid sample, respectively, were collected in a single beam mode with a 4 cm^{-1} resolution. A reference spectrum was recorded under identical scan conditions with protein-free PLGA (placebo) or buffer, as needed. The protein spectra were obtained by subtraction of the reference spectra according to the criteria described in the *Results* section. Signals for liquid and gaseous water were digitally subtracted, as previously described.[10] Final spectra are presented as second derivatives in the conformationally sensitive amide I region. Seven-point smoothing using Omnic software (Nicloet) was performed to remove white noise. To compare spectra, the second-derivative spectra were imported into GRAMS/386 (Galactic) and the area of amide I region was normalized as described previously.[2,11] Finally, the relative degree of retention of native secondary structure was determined by calculating the depth of the dominant α-helix band, relative to that for native, aqueous rhGH.

To examine protein structure in rehydrated microspheres, the samples were incubated in H_2O for 4 h prior to the measurement. For microspheres rehydrated in D_2O, 2 mg of dry microspheres were incubated with 60 μL of D_2O for at least 20 h at room temperature. Prior to measurement, the supernatant was replaced with the same volume of D_2O. This treatment was used to ensure consistent deuterium−hydrogen exchange for each sample.

Results and Discussion

The Structure of rhGH in Dry PLGA Microspheres—For storage of the final product, protein encapsulated in microspheres must be stable in the dried state. There are no published studies defining the critical criteria for storage stability of proteins encapsulated in dried microspheres. However, it is well established for freeze-dried protein formulations that retention of native protein structure is one important factor for storage stability.[3] It seems reasonable to suggest that this factor may also be the case for long-term storage stability of encapsulated proteins. Thus, a noninvasive method, such as IR spectroscopy, to assess the effect of processing and/or formulations on protein structure within dry microspheres is essential to the ultimate development of products with sufficient shelf life.

To obtain a high quality IR spectrum of protein in microspheres, it is necessary to subtract out the contribution of PLGA from the amide I region, which is used for assessment of protein secondary structure. As shown in Figure 1A, the PLGA used in the current study had only minimal absorbance in the amide I region (1600−1700 cm^{-1}). There is a large absorbance of PLGA centered around 1750 cm^{-1}, but only a slight shoulder from this band extends into the region of 1680−1720 cm^{-1}. To ensure that this small contribution from PLGA was not affecting the final protein spectra, we digitally subtracted the absorbance of PLGA microspheres without protein from the spectrum of the microspheres containing rhGH (Figure 1). A successful subtraction was indicated by the presence of a flat region at about 1730−1710 cm^{-1}. We found this criterion provided reproducible results and consistent final protein spectra, even when different investigators performed the spectral subtractions.

Figure 2 shows examples of the resulting spectra of rhGH with different formulations in dry microspheres and the spectrum of native aqueous rhGH. The native protein is primarily α-helical in structure, and hence, its spectrum is dominated by a helix band at 1656 cm^{-1}. In the spectra of the dried microsphere samples, this band is greatly reduced in intensity, indicating a loss of native helix structure. This loss is compensated by an increase in β-sheet, as evidenced by the increase absorbance at ca. 1685 cm^{-1}. The greatest reduction in helix was seen for the microspheres prepared without excipient or with mannitol. There was a slight improvement in recovery of helix content with trehalose. However, in all cases (Figure 2), the helix content of rhGH in dried microspheres was less than that

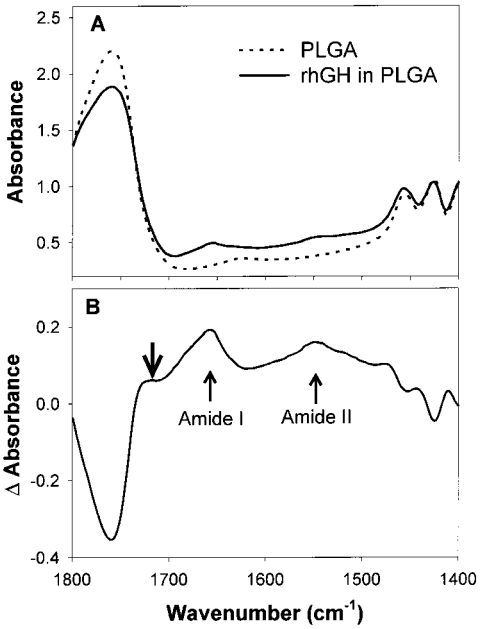

Figure 1—(A) Infrared spectra of dried rhGH in PLGA microspheres and PLGA microspheres alone. (B) The resulting spectrum of rhGH after the PLGA background was properly subtracted. The bold arrow indicates the criterion for the subtraction where a flat region should appear in the region between 1710 and 1730 cm^{-1}.

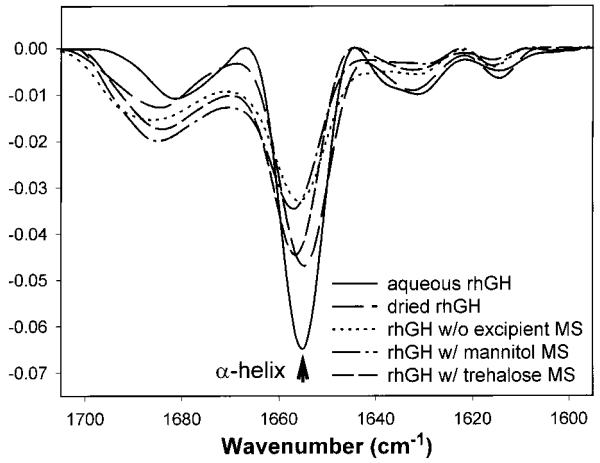

Figure 2—The second-derivative amide I spectra of dried rhGH powder and rhGH with different excipients within microspheres (MS). For comparison, the spectrum of aqueous, native rhGH in solution (measured at concentration of 20 mg/mL) is also shown. The major band at 1656 cm^{-1} is the α-helix, which is the main secondary structure of rhGH. The mannitol and trehalose formulations contained 1:1 and 0.25:1 mass ratios of excipient:rhGH, respectively, and 100 mM potassium phophate buffer, as described previously.[6]

for the protein in a lyophilized powder (prepared without excipient). This result indicates that in addition to the denaturation caused by the initial freeze-drying step, the protein structure could further be perturbed by the subsequent emulsion and encapsulation steps. Further research is needed to assess the relative effectiveness of excipients at inhibiting protein denaturation during the initial lyophilization and subsequent processing steps. This work will be necessary to obtain a final microsphere preparation with the optimal retention of native protein structure. With IR spectroscopy it should be straightforward to monitor the structure at each step.

The Structure of rhGH Microspheres rehydrated in H_2O—Although the structure of proteins in dried microspheres may be important for storage stability of the product, protein structure in rehydrated microspheres is

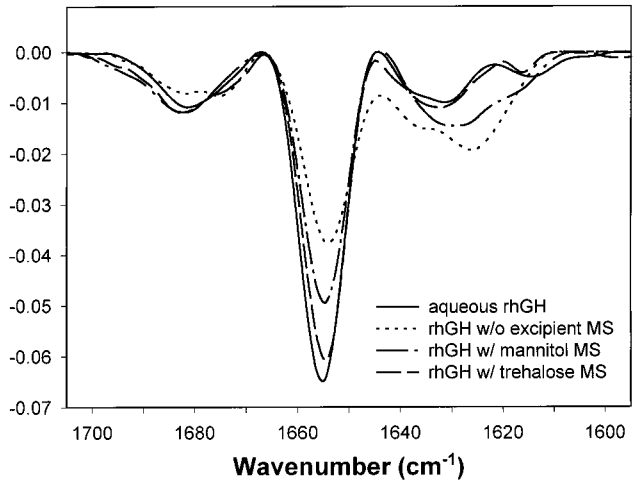

Figure 3—The second-derivative amide I spectra of H_2O-rehydrated rhGH microspheres (MS) and aqueous rhGH. The mannitol and trehalose formulations contained 1:1 and 0.25:1 mass ratios of excipient:rhGH, respectively, and 100 mM potassium phophate buffer, as described previously.[6]

most relevant to the performance of the product under physiological conditions. The structure of a dried protein is not necessarily predictive of that obtained after rehydration.[2–5,11] For example, with lyophilized protein formulations it has been found that unfolded protein molecules in the dried state may fully refold during rehydration.[2–5,11] Conversely, with some systems, it has been observed that not only is the native structure not recovered upon rehydration, but a substantial fraction of the protein population may form non-native aggregates.[2–5,11] These aggregates often have a large fraction of intermolecular beta sheet strucuture, which can be detected by specific bands in the amide I region of the IR spectrum.[2] The behavior of a given sample during rehydration depends on, at least, (1) the physicochemical properties of the protein itself, (2) the initial and final pH, (3) the effects of excipients on protein structure during the drying process and rehydration, and (4) the macroscopic properties of the dried material that dictate rates of rehydration and dissolution of protein molecules.[2–5,11] Clearly, it is not possible to predict how all of these parameters would affect the behavior of protein molecules during rehydration of microsphere formulations. Thus, direct examination of protein structure in the rehydrated microspheres is essential.

With rehydrated samples, we found that the PLGA used in the study had minimal absorbance in the amide I region. As is the case with all aqueous protein samples, the absorbance in this region was dominated by H_2O itself. We found that the well-established methods[10] for quantitatively subtracting this contribution from spectra of protein solutions also was appropriate for spectra of rhGH in rehydrated microspheres. The criterion for a successful subtraction was a flat baseline in the region 1800–2300 cm^{-1}, which has a strong absorbance from water but no signal from protein. This approach worked well for microspheres with a protein content >1% w/w (data not shown).

Figure 3 presents some examples of the second-derivative IR spectra in the amide I region of the rehydrated rhGH microspheres with different formulations. For the trehalose formulation, the α-helix band was almost identical to that for the native protein, indicating that the protein in the microspheres had refolded during rehydration (Figures 2 and 3). In contrast, the apparent extent of recovery of native α-helix was much less for the microspheres containing no excipients or for the mannitol formulation. Furthermore, the spectra for these samples had prominent absorbances in the 1620–1630 cm^{-1} region,

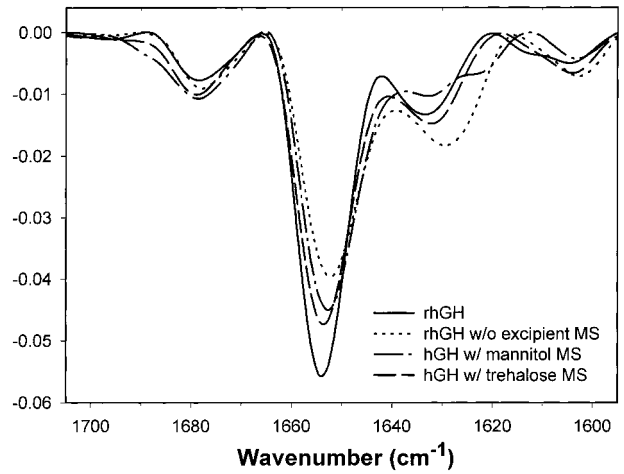

Figure 4—The second-derivative amide I spectra of D_2O-rehydrated rhGH microspheres (MS) and native rhGH in solution in D_2O. The mannitol and trehalose formulations contained 1:1 and 0.25:1 mass ratios of excipient:rhGH, respectively, and 100 mM potassium phophate buffer, as described previously.[6]

which are most likely due to the presence of intermolecular β-sheet.[2] This result indicates that a substantial fraction of the rhGH in these microsphere preparations formed aggregates during rehydration. These three examples clearly document that the behavior of proteins during rehydration of microspheres cannot necessarily be predicted from the structure of the protein in dried microspheres, as is the case for lyophilized protein formulations.

The Structure of rhGH Microspheres Rehydrated in D_2O and Its Correlation with Percent Monomer Released or Extracted from the Same Microspheres—When the protein content of microspheres was <1% wt/wt, we were not able to successfully subtract the contributions of water and PLGA from the spectrum of rhGH in microspheres (data not shown), which was, at least in part, because the concentration of protein relative to the total solution was relatively low. We have found that, even for protein solutions, the contributions of water to the amide I region cannot be subtracted quantitatively if the protein concentration is less than about 5 mg/mL (unpublished observation).

Because D_2O does not absorb strongly in the amide I region, we tested this solvent system for analyzing rehydrated microspheres. Contributions from solvent and PLGA were digitally subtracted from the spectrum for the protein in D_2O-rehydrated microspheres such that the region from 2100 to 2300 cm^{-1} was flat (data not shown).

As shown in Figure 4, the intensity of the α-helical band was again dependent on the formulation used, and we found the results were comparable overall and showed similar trends to those of H_2O-rehydrated microspheres (Figure 3). The most nativelike spectrum was seen for the trehalose formulation. The spectra for the sample prepared without excipients and that for the mannitol formulation had less intense α-helix bands and the presence of bands attributable to β-sheet at ca. 1630 and 1623 cm^{-1}, respectively.

Even microspheres with relatively low protein content could be studied in D_2O. Thus, we rehydrated all of the different formulations of rhGH in PLGA microspheres, which have previously been described,[6] in this solvent. Then, the depths of the α-helical band from the second-derivative spectra, relative to that for the native protein, were measured and used as indicators of degree of retention of native structure within the rehydrated microspheres. For each microsphere formulation, this parameter was plotted against the respective percentage of rhGH monomer recovered from either the 1-h in vitro release

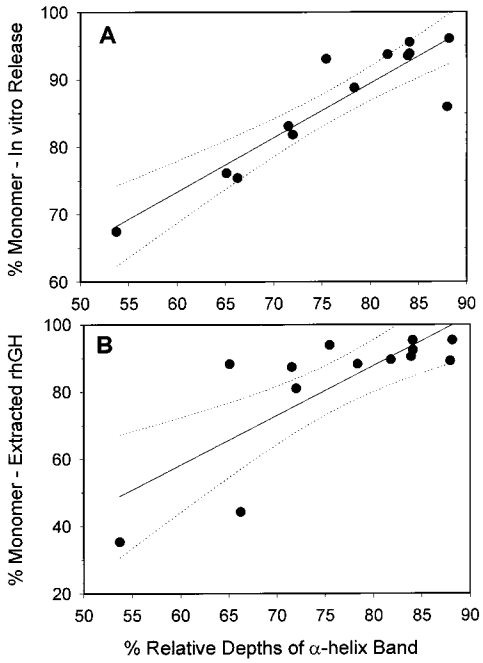

Figure 5—The linear regression analyses of the relative depth of α-helix band versus percent of recovery of rhGH monomers from either (A) 1-h in vitro release or (B) organic solvent extraction. The relations are $y = 25.1 + 0.8x$, $r^2 = 0.818$ and $y = -30.5 + 1.48x$, $r^2 = 0.618$ for A and B, respectively. The dotted lines indicate the 95% confidence intervals.

protocol or the organic solvent extraction of the microspheres (see *Methods*). As can be seen in Figure 5A, there is a strong positive correlation between the relative intensity of the α-helical band and the percent monomer obtained by the in vitro release protocol. The results of this analysis demonstrate that IR spectroscopy can not only be a valuable tool to assess the protein structure within microspheres but also may be useful to predict the integrity of protein initially released from microspheres.

Interestingly, there was a much weaker positive correlation between the relative intensity of the α-helix band and the percent of native rhGH monomer extracted from microspheres (Figure 5B). This result might reflect the fact that the organic solvent extraction of protein from microspheres could by itself furthur perturb protein structure.

The Structure of Zinc-Complexed rhGH within PLGA Microspheres—Recently, rhGH precipitated by Zn^{2+} and spray-freeze-dried has been used for the preparing PLGA encapsulated microspheres.[3] Figure 6 shows the spectra of dry rhGH prepared either from a zinc-free protein solution or from a zinc-induced precipitate (Zn-rhGH) and the spectra for dried microspheres prepared with either preparation. Both freeze-dried powders had similar spectra and almost identical loss in intensity of the α-helix band. Thus, the perturbation of rhGH secondary structure during freeze-drying was essentially the same for both preparations; zinc did not increase resistance to freeze-drying. For Zn-rhGH within microspheres, the reduction of α-helix was slightly greater than that for the freeze-dried powder, but was much less than that for microspheres prepared with non-zinc-treated rhGH. These results suggest that during the encapsulation in PLGA, the protein secondary structure could be further perturbed but that this damage is much less if the protein is initially freeze-dried as a Zn-hGH complex. More importantly, as shown in Figure 7, the Zn-rhGH within the microspheres completely refolded upon rehydration in H_2O. The spectrum for the rehydrated protein is essentially identical to that for the native, aqueous protein. Water was used in this rehydration experiment because the protein content

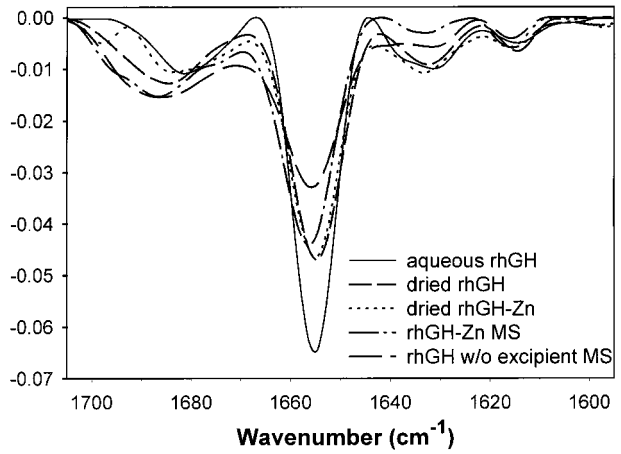

Figure 6—The second-derivative amide I spectra of dried rhGH, rhGH–Zn complex and their microspheres (MS). The spectrum of native, aqueous rhGH is also included for reference.

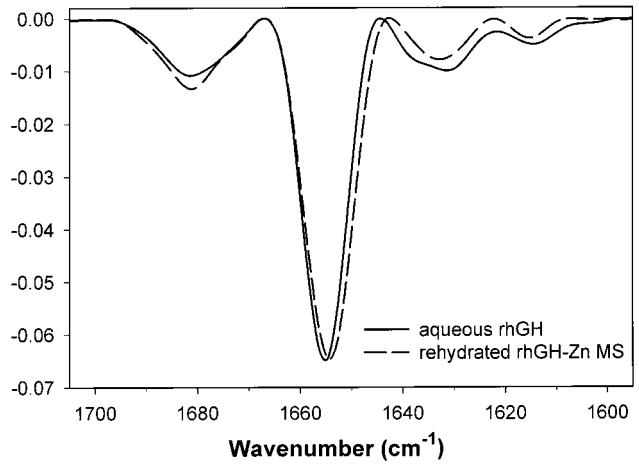

Figure 7—The second-derivative amide I spectra of H_2O-rehydrated rhGH–Zn microspheres and native, aqueous rhGH.

in these microspheres was 15% wt/wt. The refolding of the Zn-rhGH is in contrast to the aggregation of non-zinc-complexed protein during rehydration of microspheres prepared without excipients (Figures 3 and 7).

Conclusions

Noninvasive assessment of protein secondary structure in dried and rehydrated microspheres can be accomplished rapidly (e.g., <10 min is needed to acquire a high quality spectrum) with IR spectroscopy. The structure of the protein in dried microspheres may be predictive of storage stability of the protein, as is the case for lyophilized protein formulations.[3] However, more research on storage stability of proteins in dried microspheres is needed to test this suggestion. The secondary structure of protein in rehydrated microspheres is most relevant to the potential release of native, functional proteins under physiological conditions. With rhGH, the degree of retention of native secondary structure correlated directly with the percent of native monomer released during incubation in buffer. In addition, the relative effects of the method of microsphere preparation and excipients on the structure of rhGH were clearly discernible with IR spectroscopic determination of protein secondary structure. These results document that the method has utility for designing processing conditions and formulations that provide optimal retention of native protein structure in the microspheres. Finally, IR spectroscopic assessment of protein structure in microspheres could prove valuable for routine quality assurance testing of commercial lots.

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