# FT-IR and Near-Infared FT-Raman Studies of the Secondary Structure of Insulinotropin in the Solid State: $\alpha$-Helix to $\beta$-Sheet Conversion Induced by Phenol and/or by High Shear Force

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**Abstract** □ Insulinotropin (glucagon-like peptide I) is a peptide containing 31 amino acid residues. It stimulates the secretion of the hormone insulin. The solubility of this peptide is highly dependent on its environment and the treatment that it has undergone. For instance, synthetic insulinotropin is highly soluble in neutral phosphate-buffered saline (1 mg/mL). However, the application of shear force by stirring renders it extremely insoluble (1 $\mu$g/mL). This property may be explained in terms of a change in peptide secondary structure with no alteration in primary structure. In order to understand this phenomenon, FT-IR and near-IR FT-Raman were employed to examine four samples prepared under different experimental conditions. It was found that solubility decreases as the $\alpha$-helix is converted to an antiparallel $\beta$-sheet structure.

## Introduction

Proteins and peptides are often most soluble under conditions mimicking their natural environment. When they are exposed to unnatural environments, they tend to form precipitates or aggregates as a result of decreasing solubility. The physical basis for protein insolubility is still unclear. However, it has been proposed that the hydrophobicity of amino acids,[1-6] the primary structure, and the peptide backbone conformation[7-8] all contribute toward the solubility of the protein. The peptide backbone conformation may be indicative of the solubility of the protein. Many studies have suggested that protein insolubility may depend on the content of the $\beta$-sheet structure. For instance, insulin forms insoluble fibrils when heated in acid. Structural studies carried out by Burke and Rougvie[9] showed that the fibrils had a cross-$\beta$-structure. It is well-known that a $\beta$-peptide is a major component of amyloid deposits in Alzheimer's disease.[10-12] Analysis of a protein that was precipitated by salting-out also demonstrated a correlation between increased $\beta$-sheet structure and decreased solubility.[13]

Glucagon-like peptide I (insulinotropin) is a 31-amino acid peptide,[14-17] the primary sequence of which is shown in Figure 1. The solubility of insulinotropin amorphous materials varies from 1 mg/mL to 1 $\mu$g/mL in neutral pH phosphate-buffered saline depending on the method of preparation. For a better understanding of what causes the dramatic changes in solubility of insulinotropin, characterization of the secondary and tertiary structures of these solid materials is essential. FT-IR and Raman spectroscopy are the most widely used techniques to characterize protein structures in the solid state, especially when the material is amorphous. In order to examine the relationship of the $\beta$-sheet content to solubility, the structure of insulinotropin was studied in its highly soluble form and in its insoluble form using FT-IR and Raman spectroscopy.

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1 5
His - Ala - Glu - Gly - Thr - Phe - Thr -
10
Ser - Asp - Val - Ser - Ser - Tyr - Leu -
15 20
Glu - Gly - Gln - Ala - Ala - Lys - Glu -
25
Phe - Ile - Ala - Trp - Leu - Val - Lys -
30
Gly - Arg - Gly

**Figure 1**—Primary structure of insulinotropin.

Our study showed that the percentage of $\beta$-sheet conformers played a role in determining its solubility in aqueous media.

## Experimental Section

**Materials and Methods**—Insulinotropin (lot #501713) used throughout this study was synthesized by solid-state peptide synthesis and was obtained from Pfizer, Inc. Dulbecco's phosphate-buffered saline (PBS) was purchased from Gibco (Life Technologies, Inc.). Phenol (fused, USP grade) was obtained from J. T. Baker.

Sample A was the original synthetic insulinotropin, which was readily soluble. A 1 mg/mL insulinotropin solution in water for injection was stirred vigorously with a magnetic stir bar for 24 h to form aggregates. The aggregates were isolated by centrifuging the sample to pellet the solids and removing the supernatant. These isolated solids were labeled as sample B. For the preparation of sample C, a 2 mg/mL insulinotropin solution in PBS and a 4.4 mg/mL phenol solution in PBS were combined in a 1:1 volume ratio in a glass vial. The vial contents were inverted several times to mix the two solutions thoroughly. A precipitate formed immediately. However, the vial was sealed and kept at ambient temperature for 24 h to ensure complete precipitate formation. The precipitates were isolated by centrifuging the sample to pellet the solids and removing the supernatant. These isolated solids were labeled as sample C. Sample D was prepared in the same manner as sample C except that its contents were stirred vigorously for 24 h with a magnetic stir bar prior to centrifugation.

The equilibrium solubility for samples A–D was determined at room temperature by dispersing an excess amount of solid into PBS with a vortex mixer for approximately 10 s. This dispersion was allowed to sit at ambient temperature for 24 h. The dispersion was centrifuged to pellet the solids. The supernatant was filtered through a 0.22-$\mu$m Millipore Millex-GV filter and assayed by HPLC for insulinotropin concentration.

Analytical HPLC assay of insulinotropin was conducted on a Vydac Protein C4 column (Rainin Cat# 214TP54). The gradient program used for the assay is listed below

| time (min) | 0 | 5 | 30 | 35 | 37 | 46 |
|---|---|---|---|---|---|---|
| %A | 75 | 75 | 50 | 50 | 75 | 75 |
| %B | 25 | 25 | 50 | 50 | 25 | 25 |

where A represents 0.1% trifluoroacetic acid (TFA) in water and B represents 0.1% TFA in acetonitrile. The flow rate was set at 1 mL/min



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**Table 1—Equilibrium Solubility of Samples A–D in pH 7.1 Phosphate-Buffered Saline**

| Samples | Solubility (mg/mL) | Samples | Solubility (mg/mL) |
|---|---|---|---|
| A | 1 | C | 0.47 |
| B | 0.001 | D | 0.001 |

and the column kept at ambient temperature (25 °C). Detection was by UV at 215 nm. Insulinotropin retention time was ca. 23 min.

The equipment used for the HPLC assay consisted of an LDC Consta-Metric 4100 solvent delivery system, a Bio-Rad Model AS-100 HRLC automatic sampling system, an LDC SpectroMonitor 4100 programmable variable-wavelength detector, and a Spectra-Physics Chrom Jet integrator.

**Vibrational Spectroscopy**—FT-IR spectra of proteins were obtained by microinfrared measurements, using an IR-MAU100 unit with a MCT detector (IR-DET102) attached to a JEOL JIR-6500 FT-IR spectrometer.[18] All the spectra were recorded at a 4-$cm^{-1}$ resolution with 200–1000 scans. The proteins were put on a Ge window and crushed to obtain satisfactory spectra. The second-derivative spectra[19] were calculated (smoothing points, 13) by using JEOL JIR-6500 FT-IR software. The percentage contributions of the $\alpha$- and $\beta$-conformations were calculated on the basis of the method of Dong et al.[20] All these calculations have been made using the area under the peaks from the second-derivative spectrum.

Near-IR FT-Raman spectra of proteins were measured at an 8-$cm^{-1}$ resolution with a JEOL JRS-FT 6500N Raman spectrometer equipped with an InGaAs detector.[21] The 1064-nm line of a CW Nd:YAG laser (Spectron SL301) was used for excitation, and the laser power employed at the sample position was 350–850 mW. Raman scattering light was collected with a 180° backscattering geometry, and all the spectra were the result of the coaddition of 2000–4000 interferograms.

**Hydrophobicity Analysis**—Hydrophobicity analysis of this peptide was possible since the primary sequence was known. The Kyte and Doolittle scale of hydropathy was used to evaluate the hydrophilic and hydrophobic regions of the peptide.[22] This method uses a moving-segment approach that continuously determines the hydropathy within a segment of predetermined length ($n$) as it advances through the sequence from the amino to the carboxy terminus.[22] Six is often chosen as $n$, so that the segment length is larger than the oscillations associated with the periodic helix or strand and yet sufficient local information is retained. For insulinotropin (31-amino acid peptide), an average of hydropathy over six residues was calculated up to the 26th residue, and then the $n$ was reduced by one each time as the C-terminal was approached.

**Circular Dichroism (CD) Measurements**—CD spectrum was recorded on a J-720 Jasco circular dichroic spectropolarimeter in 0.01-cm pathlength cells at 25 °C. The peptide concentration was 0.8 mM in a 50 mM phosphate buffer at pH 8.

**Water Content Determination**—Water contents in the solid samples were determined by two different methods: coulometric Karl Fisher titration[23] (Mitsubishi moisture meter, Model CA-06, Mitsubishi Kasei Corp., Tokyo, Japan) and thermogravimetric analysis[24] (TGS-2 analyzer, Perkin-Elmer, Norwalk, CT).

## Results

**Solubility Behavior**—The equilibrium solubility in PBS for samples A–D are summarized in Table 1. The solubility of the original synthetic insulinotropin (sample A) is 1 mg/mL. If an insulinotropin solution in water or PBS is stirred vigorously, highly insoluble aggregates are formed. The isolated solids (sample B) have a solubility of about 1 $\mu$g/mL in PBS. The solubility of the phenol precipitate of insulinotropin (sample C) is 0.47 mg/mL in PBS. Sample D constitutes the aggregates isolated from an insulinotropin solution and a phenol solution that were combined and then stirred overnight. This sample has a solubility of about 1 $\mu$g/mL in PBS.

**Peptide Backbone Analysis by FT-IR**—The band assignments for different conformations are studied by many investigators are summarized in Table 2.[25,26]

**Table 2—IR Band Assignment of Amide I and II to Different Conformers of Polypeptide Studied by Other Investigators[25,26]**

| Conformer | Frequency ($cm^{-1}$) Amide I | Amide II |
|---|---|---|
| $\alpha$-Helix | 1650–1655 | 1516–1546 |
| Antiparallel $\beta$-sheet | 1632–1635 | 1530 |
| | 1668 (theoretical) | |
| | 1685 | |
| Parallel $\beta$-sheet | 1630 | 1530–1550 |
| | 1645 | |
| $\beta$-Turn | 1685–1690 | Not studied |
| $^{3}10$ Helix or distorted $\alpha$-helix | 1660 | Not studied |

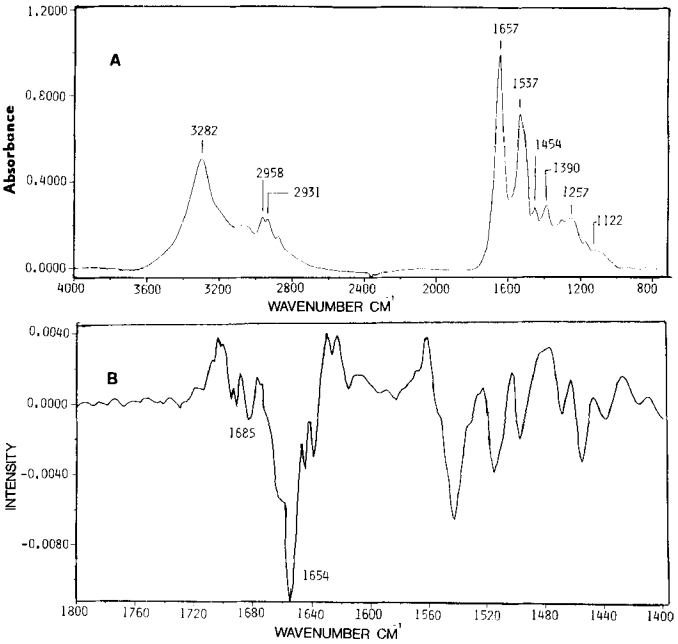


**Figure 2**—(A) FT-IR spectrum of synthetic insulinotropin (sample A). (B) Second-derivative of sample A.

The FT-IR spectra of the solid insulinotropin samples (A, B, C, and D) made under different conditions are shown in Figures 2A, 3A, 4A, and 5A, respectively, and their frequencies in the amide I and II regions are summarized in Table 3.

Instead of relying only on the original FT-IR spectra, the second-derivative spectrum of each sample was also obtained (Figures 2B, 3B, 4B, and 5B, respectively) since small shoulders in the spectrum can be seen as clear peaks in the second-derivative spectrum. The area under these peaks can be used to determine the percentage composition of the various structures.[19,20] The contents of different conformers for samples A–D were obtained by analyzing Figures 2B, 3B, 4B, and 5B, and are summarized in Table 4. The differences in the amide regions of the spectra of the four samples are very clear and distinct. The peak at 1654 $cm^{-1}$ is strong and distinct only in the spectrum of sample A. Sample B shows an increase in intensity of the peak at 1668 and 1635 $cm^{-1}$. Sample C has a very intense peak at 1635 $cm^{-1}$. Sample D, on the other hand, has two very intense peaks at 1668 and at 1632 $cm^{-1}$. These clear differences can allow us to draw valid conclusions on structural differences among the four samples.

For sample A, a major peak was observed at 1657 $cm^{-1}$ (Figure 2A). This is an indication of an $\alpha$-helix.

The corresponding second-derivative spectrum reveals much more information (Figure 2B). The main peak is at 1654 $cm^{-1}$, which is an indication of an $\alpha$-helix (49%). The 1685-$cm^{-1}$ band, shown in the second-derivative spectrum as a small but distinct

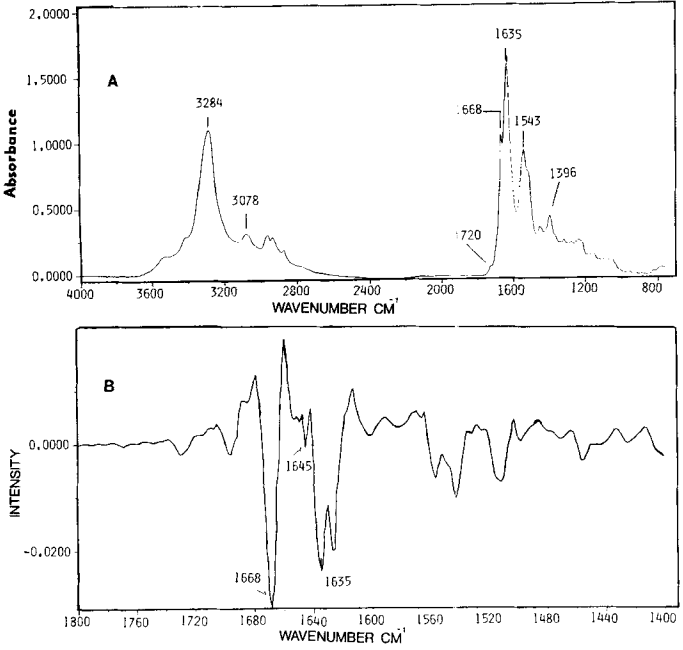


**Figure 3**—(A) FT-IR spectrum of sample B. (B) Second-derivative spectrum of sample B.

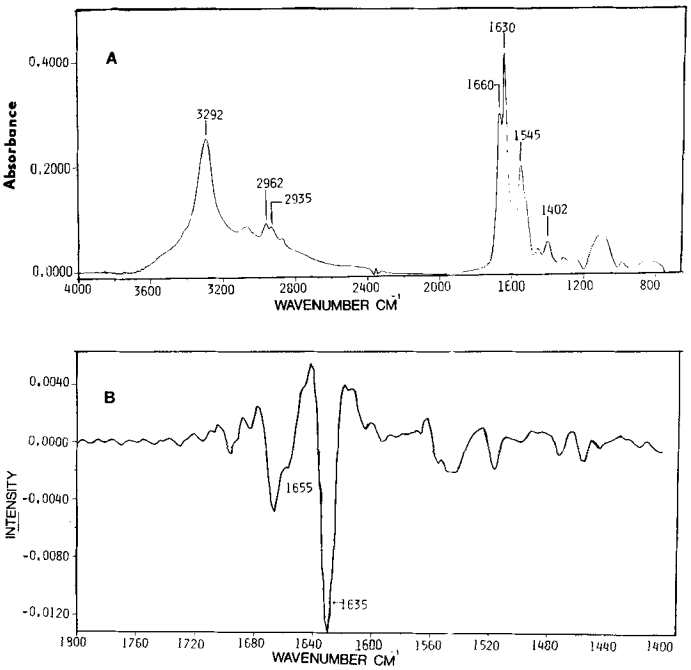


**Figure 4**—(A) FT-IR spectrum of sample C. (B) Second-derivative spectrum of sample C.

peak, indicates the presence of a small amount of an antiparallel $\beta$-sheet or $\beta$-turn.

Sample B shows dramatic changes in its FT-IR and second-derivative spectra as compared to sample A. The $\alpha$-helix band at 1657 $cm^{-1}$ is no longer visible (0% $\alpha$-helix). Moreover, the amide I band has two peaks at 1635 $cm^{-1}$ (major) and at 1668 $cm^{-1}$ (minor) in the FT-IR spectrum (Figure 3A). The second derivative spectrum (Figure 3B) also clearly indicates the presence of bands at 1635 and 1668 $cm^{-1}$. Both of the 1635 and 1668 $cm^{-1}$ bands are indications of an antiparallel $\beta$-sheet structure. A small band at 1645 $cm^{-1}$ is observed in the derivative spectrum, but not in the original IR spectrum. The spectra of Figure 3A,B clearly signify that there is a dramatic change in the

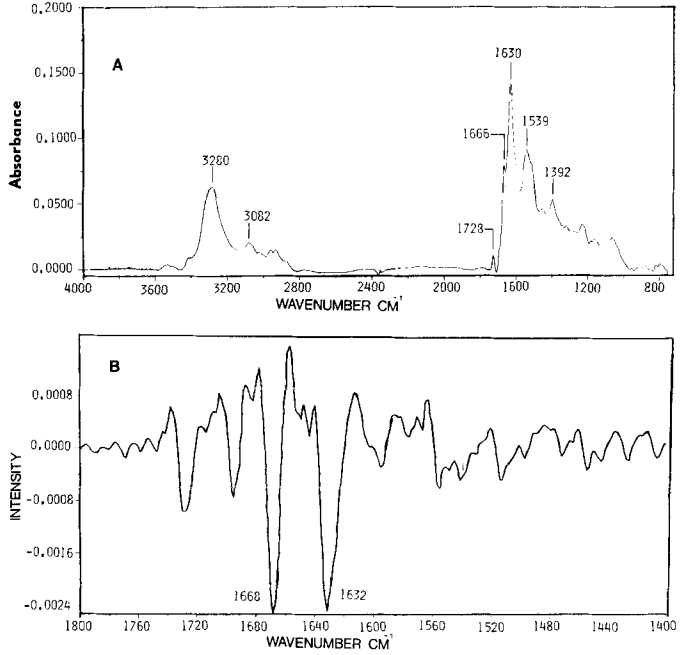


**Figure 5**—(A) FT-IR spectrum of sample D. (B) Second-derivative spectrum of sample D.

**Table 3—Frequency Observed for Insulinotropin Samples A–D in Amide I and II Regions Studied by FT–IR**

| Sample | Frequency ($cm^{-1}$) | |
|---|---|---|
| | Amide I | Amide II |
| A | 1657 | 1537 |
| B | 1635 (major)<br>1668 (minor) | 1543 |
| C | 1630 (major)<br>1660 (minor) | 1545 |
| D | 1630 (major)<br>1666 (minor) | 1539 |

backbone conformation when insulinotropin is subjected to high shear force.

Sample C is the precipitate formed when an insulinotropin solution is combined with phenol solution. The IR spectrum of sample C is basically similar to that of sample B with peaks at 1630 and 1660 $cm^{-1}$ (Figure 4A). The presence of these antiparallel $\beta$-sheet bands is also seen in the second-derivative spectrum (Figure 4B). However, in contrast to sample B, a shoulder was observed at 1655 $cm^{-1}$ (Figure 4B). This is an indication of an $\alpha$-helix structure, which was quantitated to be 21% of the overall structure.

Sample D was prepared by applying shear force to sample C before it was isolated from the mother liquor. The FT-IR spectrum of sample D (Figure 5A) is very much like those of samples B and C. However, the second-derivative spectrum (Figure 5B) is more similar to that of sample B (Figure 3B) than sample C (Figure 4B). For sample B, there are two bands in the 1632-$cm^{-1}$ region, instead of one band as for sample D.

There are broad bands in the region of 2800–3100 $cm^{-1}$ in the spectra of all samples (Figure 2–5). These bands are constituted of C–H stretching vibration, overtones of C–H bending, and Fermi resonance bands. The interpretation of these bands would be very important, if the samples were lipids or membranes. However, no correlation was reported between the secondary and tertiary structures of proteins and these bands. Therefore, no further analysis was carried out on these bands. The distinct

**Table 4—Characteristic Band Frequencies and the Content of Different Conformers for Insulinotropin Solids Prepared by Different Methods**

| Sample | Frequency ($cm^{-1}$) | Conformation Assignment | Content (%) |
|---|---|---|---|
| A | 1654 (very large) | $\alpha$-Helix | 49 |
| | 1635 | Antiparallel $\beta$-sheet | 13 |
| | 1685 (small) | Antiparallel $\beta$-sheet | 12 |
| | | Random coil[a] | 26 |
| B | 1635 (large) | Antiparallel $\beta$-sheet | 22 |
| | 1668 (very large) | Antiparallel $\beta$-sheet | 32 |
| | | Random coil[a] | 46 |
| C | 1630 (very large) | Antiparallel $\beta$-sheet or parallel $\beta$-sheet | 49 |
| | 1655 (shoulder) | $\alpha$-Helix | 21 |
| | 1666 (broad and moderate size) | Distorted $\alpha$-helix or antiparallel $\beta$-sheet | 37 |
| | | Random coil[a] | 0 |
| D | 1632 | Antiparallel $\beta$-sheet or parallel $\beta$-sheet | 39 |
| | 1666 | Antiparallel $\beta$-sheet | 27 |
| | | Random coil[a] | 34 |

[a] The content of random coil is calculated from the difference between the total content and the summation of $\alpha$-helix and $\beta$-sheet structure contents.

peak at 3280–3290 $cm^{-1}$ is obviously from OH vibration of the water molecules present in the sample and the NH vibration of the amino groups of the peptide. In all of these samples the amide II band is very distinct (1539–1545 $cm^{-1}$). This amide II band is Raman inactive and thus is not seen in the Raman spectra (Figure 6).

**Near-Infrared FT-Raman Spectroscopy**—Raman spectroscopy has been employed extensively for protein conformation identification for the last 2 decades. Recently near-infrared FT-Raman spectroscopy has been rapidly developed, as it has advantages over conventional Raman spectroscopy. Near-infrared excitation can avoid fluorescence background and largely reduce photodecomposition. Therefore, FT-Raman spectroscopy provides Raman spectra with highly accurate frequencies with clean backgrounds.[27] For the present study, the amide I and III regions were examined carefully.[28–30]

For the $\alpha$-helix conformation, the amide I frequency is relatively low, ranging from 1642 to 1658 $cm^{-1}$. The antiparallel $\beta$-sheet structure is in the higher frequency range of 1662–1680 $cm^{-1}$. Frequencies due to random coil structures are between the frequencies of the $\alpha$-helix and $\beta$-sheet, i.e., between 1660 and 1665 $cm^{-1}$. The amide III band of the $\alpha$-helix appears at 1260–1300 $cm^{-1}$, while that of the random coil has relatively low frequencies of 1240–1250 $cm^{-1}$. For the $\beta$-sheet structure, the amide III absorbs at 1242–1260 $cm^{-1}$.

The original synthetic insulinotropin (sample A) gives a distinct amide band at 1659 $cm^{-1}$, indicating the presence of an $\alpha$-helical conformation (Figure 6A). Although the main peak is located at 1659 $cm^{-1}$, there are shoulders at 1685 and 1695 $cm^{-1}$. This suggests that the secondary structure of sample A consists mainly of an $\alpha$-helix, but some amount of $\beta$-sheet is also present in the native, untreated insulinotropin.

For the amide III region, there are continuous ascending bands from 1250 to 1300 $cm^{-1}$. This region is a highly mixed vibrational zone. For an accurate determination of amide III bands, it is usually recommended to dissolve the protein of interest in $D_2O$ to allow isotopic exchange. The deuterated protein should give a new amide III′ band in the vicinity of 980 $cm^{-1}$, and any bands not shifted in the region of 1200–1300 $cm^{-1}$ are not amide III bands.[28,29] Isotopic exchange of hydrogen and deuterium atoms induced by dissolving sample A in $D_2O$ caused these bands to disappear and shift to lower frequencies (the spectrum is not

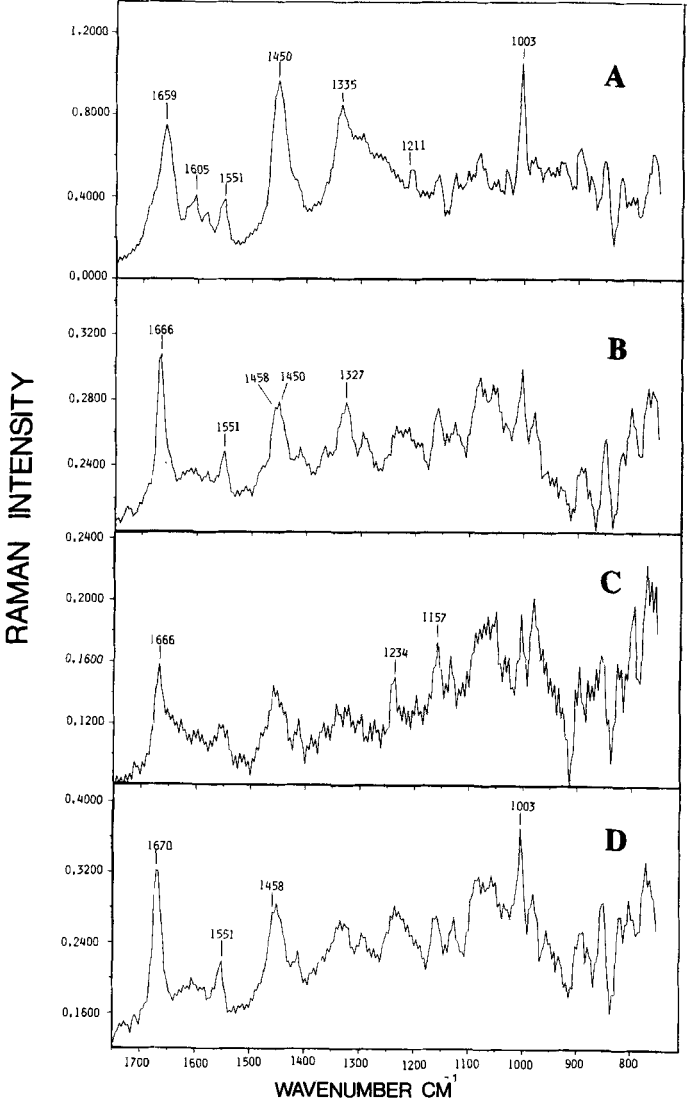


**Figure 6**—FT-Raman spectra of insulinotropin in the solid state: (A) sample A, (B) sample B, (C) sample C, (D) sample D.

shown). This suggests that the continuous ascending bands in 1250–1300 $cm^{-1}$ are an expression of an $\alpha$-helix.

Due to the low solubility of samples B–D, hydrogen/deuterium isotopic exchange could not be carried out. The Raman spectra of samples B–D are shown in Figure 6B–D.

The amide I bands of sample B–D are quite similar to each other: 1666 $cm^{-1}$ for sample B, 1666 $cm^{-1}$ for sample C, and 1670 $cm^{-1}$ for sample D. This is an indication of an antiparallel $\beta$-sheet structure for these samples. Resolution of the amide I band for Raman is not as good as that for FT-IR, but it clearly shows that there is a shift of $\alpha$-helix to $\beta$-sheet structure from sample A to sample B–D. The decrease in solubility (Table 1) from sample A to samples B–D can be correlated with a major conformation change from $\alpha$-helix to $\beta$-sheet structures.

The C–H bending vibrational mode is strongly Raman active, and an intense Raman band is observed at 1450 $cm^{-1}$ in all samples. However, this band is very weak in the IR spectra (Figures 2–5)

**Hydrophobicity Analysis**—The hydrophobicity analysis of insulinotropin (Figure 7) indicated that the region Ala(18)–Ala-(24) is the most hydrophobic. This hydrophobic region is located between two hydrophilic regions, Ser(11)–Gln(17) and Trp(25)–Gly(31). The His(1)–Val(10) is neither very hydrophobic nor very hydrophilic. These regions would therefore determine the

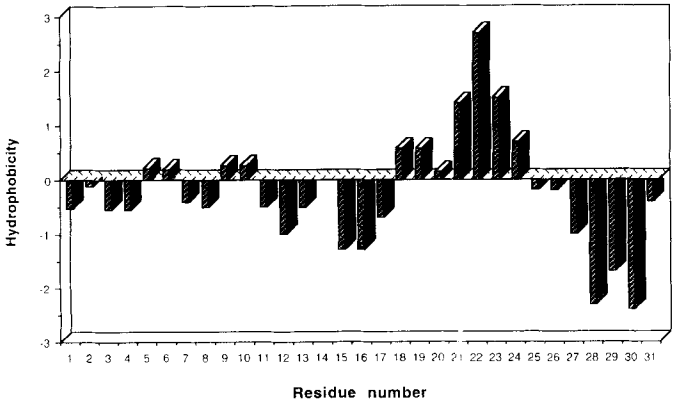


**Figure 7**—Hydrophobicity of insulinotropin. The values were calculated by the Kyte and Doolittle method.[22]

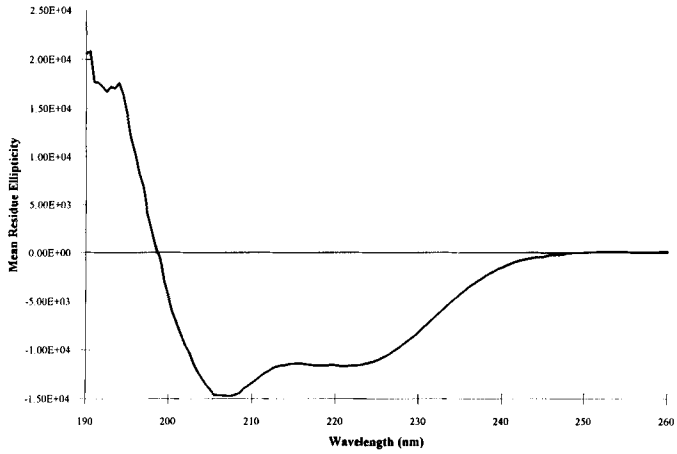


**Figure 8**—Far-UV CD spectrum of insulinotropin in solution. The peptide concentration was 0.8 mM in a 50 mM phosphate buffer at pH 8.

manner in which the peptide would fold under different environmental conditions such as solvent, pH, and ionic strength.

**Circular Dichroism**—Figure 8 shows the far-UV CD spectrum of insulinotropin in solution. The spectrum is characteristic of an $\alpha$-helical conformation: two minima at 207 and 223 nm and a maximum at near 190 nm.[31]

**Water Content Determination**—Sample A was the only dry powder, and samples B–D were wet cakes. Water content determinations were performed in duplicate for each sample. The two methods, thermogravimetric analysis and coulometric Karl Fisher titration, showed almost the same results, although the former tended to give a little higher (0.3% higher on an average) water content values than the latter. The water content of sample A was 6.8% w/w, and those of the wet samples (B–D) were in the range of 85–87% w/w.

## Discussion

Insulinotropin is composed of 31 amino acid residues. The hydrophobicity profile indicates that the highest hydrophobicity region lies between Ala(18) and Ala(24). The original synthetic insulinotropin (sample A) has a high solubility in aqueous media with a high $\alpha$-helix content, as demonstrated in the CD spectrum (Figure 8). It suggests that the Ala(18)–Ala(24) hydrophobic region must be buried inside by the molecule and the hydrophilic regions must be in the exposed state. We can presume that the two hydrophylic regions, Ser(11)–Gln(17) and Trp(25)–Gly(31), form $\alpha$-helixes and are in good contact with water molecules.

Prestrelski et al. reported dehydration-induced conformational changes in proteins.[32] They observed conformational transitions from a helical conformation or an unordered structure to a $\beta$-sheet after freeze-drying protein solutions. Similarly, we observed an $\alpha$-helix to $\beta$-sheet transition when the original peptide (sample A) in solution was exposed to phenol (sample C) or high shear force (samples B and D). Samples A was the only dry powder (moisture content = 6.8% w/w), and the other samples (B–D) were not subjected to any kind of drying process besides centrifugation (moisture contents $\geq$ 85% w/w). Therefore, it is difficult to relate the cause of the $\alpha$ to $\beta$ conversion observed in our study to dehydration.

Ismail et al. demonstrated that certain denaturing conditions could cause irreversible intermolecular self-association of a peptide that was accompanied by the appearance of a very strong IR absorption band at 1618 $cm^{-1}$.[33] Since we have not observed such a band in the 1610–1620 $cm^{-1}$ region of the IR spectra for any of the insulinotropin samples, it is unlikely that the decreased solubility for the precipitated samples is attributed to irreversible self-association. The high-shear-induced precipitates (samples B and D), which have the lowest solubility in neutral pH range (1 $\mu$g/mL solubility in PBS or water), are in fact readily soluble in an acidic solvent such as 0.01 N HCl or 0.1% trifluoroacetic acid in water. We have performed an in vitro bioactivity test[34] with the high-shear-induced precipitates of insulinotropin after redissolving them in an acidic solvent. No differences in bioactivity were observed between the original peptide (sample A) and the precipitated samples, and all of them were fully active.

The self-association of synthetic insulinotropin in solution was characterized by Grucza et al. using equilibrium analytical ultracentrifugation and CD spectroscopy.[35] It was determined that the peptide formed a tetrameric species with significantly higher $\alpha$-helical content than the monomer. They also observed that the tetramers precipitated out of the solution upon storage at ambient temperature overnight, but the aggregation was completely reversible.

It has been suggested that proteins in solution are destabilized by protein adsorption at hydrophobic interfaces (air–water interface or water–container materials), and that the initial step is nucleation. Partially or completely unfolded protein molecules form nuclei which serve as precursors to large aggregates.[36–40] Since insulinotropin does not contain any disulfide bonds, the peptide may not have a tightly packed three-dimensional structure. With shaking or stirring, the air–liquid and the liquid–solid interfaces increase, leading to greater conformational changes and denaturation. From the results in Table 4, it is evident that insulinotropin underwent conformational changes when it was subjected to vigorous agitation. For the samples B and D, the contents of the $\beta$-sheet and random coil increased at the expense of the $\alpha$-helix. The mechanism of this conformational conversion is not understood, however. This may not be a single event.

The helix to random coil transition is one of the most thoroughly studied phenomena. Interconversion of the two structures can occur upon changing the pH, the solvent, the temperature, etc., and the transition is very abrupt, indicative of a cooperative system.[41] However, the mechanism of $\beta$-sheet formation is not well understood. Synthetic polypeptides that form $\beta$-sheets generally produce large, insoluble structures comprising many peptide chains.[41] With the limited information available, one can propose two separate events occurring.[42] At first, insulinotropin molecules lose their helical structure and become disordered due to the hydrophobic interfaces. Then, the disordered chains aggregate, forming a $\beta$-sheet structure, which results in a low solubility in aqueous media. A kinetic study is being carried out in our laboratory to elucidate the mechanism of this phenomenon.

When insulinotropin is mixed with phenol without stirring (sample C), the solubility is still low (470 $\mu$g/mL), but much higher than that of samples B and D, which were stirred (1 $\mu$g/mL). Both IR and Raman spectroscopy provide evidence for

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