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Solution conformation of model polypeptides with the use of particle beam LC/FT-IR spectrometry and electrospray mass spectrometry

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Abstract

Solution conformations of the polypeptides β endorphin (β-END) and a cysteine peptide (CYSP) were investigated with the use of particle beam LC/FT-IR spectrometry. Gradient elution HPLC with mobile phases that contained acetonitrile with 0.1% TFA (v/v) and 0.1% aqueous TFA (v/v) were used. The conformations of both polypeptides were studied in 0.9% sodium chloride injection USP, 5% dextrose in water injection USP and sterile water for injection USP. Additional conformational studies over a pH range of 2–10, temperatures of 25, 50, 75 and 100°C and after storage for 24 h were investigated. The studies indicated that the two polypeptides did not behave similarly under identical conditions. It was observed that both β-END and CYSP had slightly different conformations in the various parenteral solutions. It was also shown that the conformation of CYSP changed with both pH and temperature while β-END was conformationally stable to both temperature and pH. The identity of the peptides and the conformationally sensitive charge-state intensities of the peptides were investigated with electrospray ionization mass spectrometry (ESI/MS). The combination of IR and MS data allowed an estimation of solution effects on the conformations of the model polypeptides.

Keywords: Conformation; Peptides; Particle beam LC/FT-IR spectrometry; Electrospray MS; HPLC; β endorphin; CYSP; Charge-state intensities

1. Introduction

The ineffectiveness of traditional medicines to cure infectious diseases has led to the widespread interest in the discovery and characterization of biologically based therapeutic and diagnostic drugs [1]. Characterization of these biopharmaceuticals has been an issue of recent concern [2]. The complex higher-order structure of peptides, polypeptides and proteins pose problems in their purification, storage and administration [3,4]. A

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loss in the higher order structure may lead to a change in the pharmacological activity. Peptides and polypeptides are susceptible to both physical and chemical changes in the gastro-intestinal tract and hence must be administered intravenously. Chemical changes are usually monitored with mass spectrometry [5] or amino acid sequencing, but there is no defined methodology to monitor the conformational changes of biopharmaceuticals in aqueous solution.

High performance liquid chromatography (HPLC) is a well established and reliable technique for analysis of peptides and their impurities [6–9]. Ultraviolet detection at 220/215 nm and fluorescence detection after pre/post column derivatization have been traditionally used for peptides, polypeptides and proteins [10–12]. More recently, mass spectrometry (MS) has become a very important tool in the structural characterization of these compounds [5,13,14] and LC-MS has been routinely used in such applications as the characterization of tryptic digests [15].

Infrared spectrometry (IR), as a stand alone technique, has been used for conformational analysis of peptides and proteins [16]. The secondary structural information is primarily contained in the amide I band (1600–1700 cm^{-1}) [17] which is extremely sensitive to conformational changes. Lately, there has been interest in the use of LC/FT-IR interfaces for the analysis of pharmaceuticals and other products [18–20]. The particle beam (PB) LC/FT-IR interface, developed at the University of Georgia, is a low temperature and pressure solvent elimination apparatus that restricts the conformational motion of proteins [21–24]. The interface has recently been used to study the conformational states of chromatographically analyzed proteins [25,26]. Heterogeneity of samples has limited the use of FT-IR in stability studies. The degradation of peptides leads to the formation of smaller peptide fragments which can interfere with the IR spectrum of a sample. In formulations that contain biopharmaceuticals, there are a number of preservatives that can affect the conformation of the peptide. Hence, there is a need to remove these preservatives prior to the conformational analysis of the peptides.

Recently, higher-order structures of proteins have been investigated with MS [27–29]. Electrospray ionization (ESI), a soft ionization technique, has been shown to produce intact multiply-charged gas phase ions from protein molecules in solution [28–30]. Amide hydrogen/deuterium exchange rate measurement by ESI/MS has been used as an analytical technique to probe protein secondary structure. This technique was developed over the last few years and is limited in that only approximate estimates of α-helical, β-sheet and random coil structures can be obtained [27,31].

The conformational effects of different HPLC columns on various forms of bovine ribonuclease A were investigated with the use of particle beam LC/FT-IR spectrometry and electrospray ionization [32]. Solution conformation of angiotensin I, a decapeptide and POMC-X, a N-terminal octapeptide fragment of pro-opiomelanocortin were also investigated with PB LC/FT-IR and ESI/MS The data indicated that the technique could be used as a tool for the analysis of small peptides with 8–10 residues. The objective of this study was to further investigate the use of PB LC/FT-IR as an analytical technique to study the conformational changes of polypeptides in solution. The β-endorphin (β-END) and CYSP, 31 and 21 residue polypeptides, respectively, were chosen as model compounds. They were reconstituted in various parenteral mixtures such as 0.9% sodium chloride injection USP, 5% dextrose in water injection USP (D5W) and sterile water for injection USP and the solution effect on the conformation of the peptides was investigated. The effects of pH, temperature and time on the conformation of the model polypeptides in aqueous media were studied. Electrospray MS was used as an additional technique to PB LC/FT-IR. Studies have indicated that PB LC/FT-IR can be used effectively to identify conformational changes in model polypeptides. Studies also indicated that the data obtained with ESI/MS correlates well with the IR data obtained with PB LC/FT-IR.

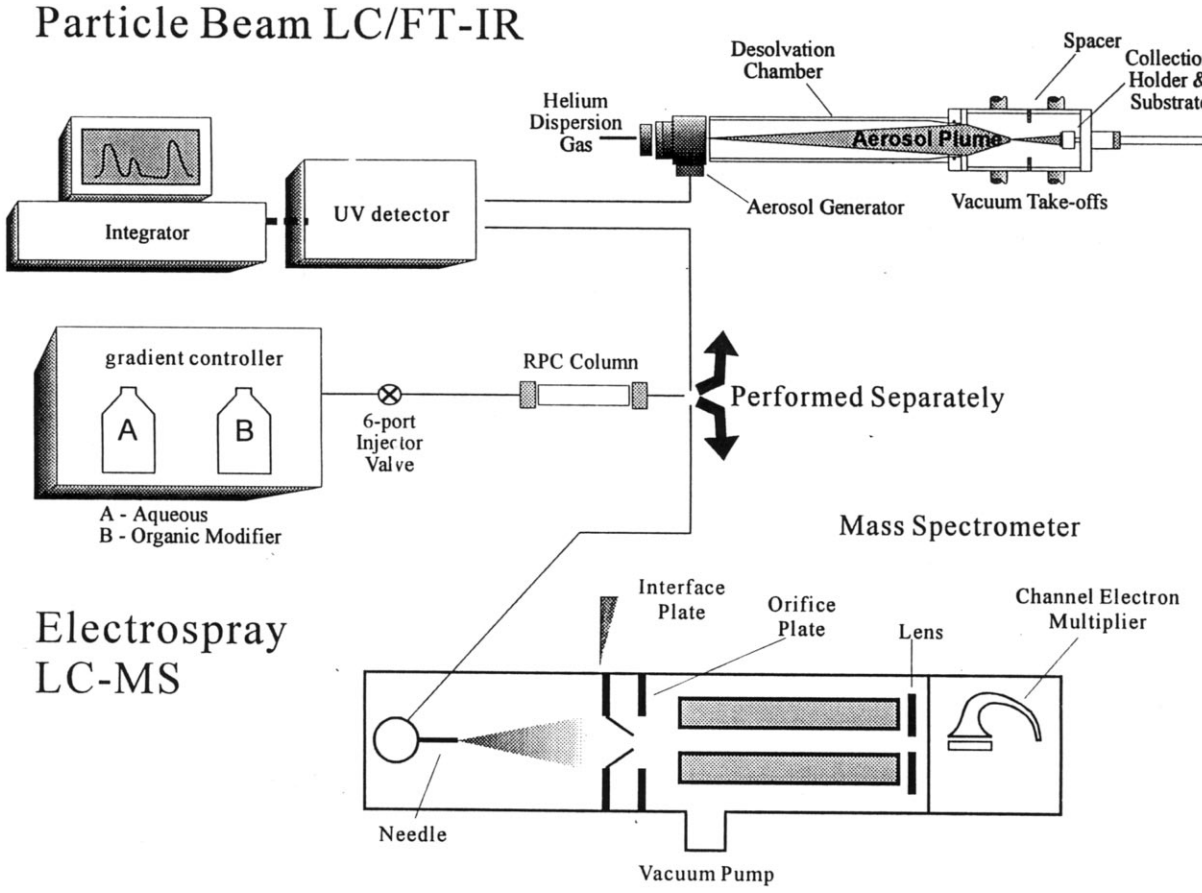

Fig. 1. Schematic of the particle beam LC/FT-IR spectrometer and the electrospray LC-MS.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of tandem Varian model 2510 HPLC pumps (Walnut Creek, CA) connected to a Varian static mixer. The mixer was attached to a Rheodyne model 7125 manual injector (Cotati, CA) equipped with a 100 μl loop. A Zorbax (Chadds Ford, PA) octadecylsilane analytical column (2.1 × 150 mm, 5 μm, 300 Å) was used to separate the peptides from other components. A Micromeritics (Norcross, GA) model 787 variable wavelength UV-Vis detector was used to detect the analytes. The output was recorded with a Spectra Physics model 4290 Integrator (San Jose, CA). The UV detector was connected to the particle beam interface (Fig. 1). The particle beam interface was equipped with a 22 cm desolvation chamber, a single momentum separator and a 5 cm nozzle to substrate distance. The column effluent was converted into an aerosol with 25 μm silica tubing (Polymicro Technologies, Phoenix, AZ). The column was thermostatted at ambient temperature (23°C) with the use of a column heater (Fiatron Systems, Milwaukee, WI). Desolvated peptide deposits were collected onto calcium fluoride disks (25 × 2 mm, International Crystal, Garfield, NJ) and interrogated off-line with a Spectra Tech IR-Plan™ infrared microscope (Shelton, CT) interfaced to a Perkin-Elmer 1725X FT-IR spectrometer (Norwalk, CT). All spectra were obtained from 1000 scans at 8 cm^{-1} resolution. All spectral manipulations including

second derivative and deconvolution were performed with GRAMS 386™ software (Galactic, Salem, NH). The mass spectral data were obtained with a VG Quattro II triple quadrupole mass spectrometer (Micromass, Beverly, MA). The Q2 and Q3 sections of the MS/MS system were not utilized as ion detection occurred prior to Q2 for all experiments described herein.

2.2. Reagents and Chemicals

The β endorphin (β-END) was obtained from Sigma (St. Louis, MO). CYSP was synthesized at the molecular genetics facility in the University of Georgia (Athens, GA). Sequences of the two polypeptides are shown in Fig. 2. The purity of CYSP was confirmed by HPLC to be greater than 90%. Trifluoroacetic acid (TFA) was also obtained from Sigma. Water was purified by a cartridge system (Continental Water Systems, Roswell, GA). Acetonitrile (Fisher, Pittsburgh, PA) was HPLC grade and all other reagents used in the assay were Baker Analyzed reagents.

2.3. Preparation of solutions

Individual solutions (0.2 mg ml^{-1}) of β-END and CYSP were prepared by weight of 0.2 mg of each polypeptide in individual 1 ml volumetric flasks followed by addition to volume of either 0.9% sodium chloride injection USP, D5W USP or sterile water for injection USP. Solutions of the polypeptides in the different parenteral carriers were stored at ambient temperature (23 ± 2°C) and aliquots were removed and assayed at 0 and 24 h.

A 10 mM volume of ammonium acetate solutions of pH 2.25, 4.0, 6.0, 8.0 and 10.0 were prepared for the pH studies. Either glacial acetic acid or ammonium hydroxide was used to adjust the pH. Solutions of the model polypeptides in each acetate buffer were prepared by weight with the appropriate amounts of the polypeptides in separate 1 ml volumetric flasks and ammonium acetate buffer of the different pH's were added to volume.

A : YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE

B : GSSIRYRSRCYSIRRNEFARK

Fig. 2. Amino acid sequence of β endorphin (A) and CYSP (B).

In the case of temperature studies, individual solutions of β-END and CYSP were prepared in water. Individual samples were weighed in separate 10 ml volumetric flasks and water added to volume. Separate aliquots of 2 ml were pipetted into a test tube and heated in a water bath to 50, 75 and 100°C. They were stored under the above conditions for 1 h and a deposit was obtained with the use of the particle beam interface. The deposit was then examined for changes in the conformation of the polypeptide.

2.4. Chromatographic conditions

A narrow bore Zorbax SB 300 C-18 column (2.1×150 mm) with a pore size of 300 Å and particle size of 5 μm was used for the analysis of both polypeptides. The mobile phase used was 0.1% trifluoroacetic acid (TFA) (A) and 0.1% TFA in acetonitrile (B). A gradient program of 20–100% B over 40 min at a flow rate of 0.25 ml min^{-1} was used for the analysis of β-END. An injection volume of 100 μl and a detection wavelength of 220 nm was used to detect the analyte. A gradient program of 10–100% B over 40 min at a flow rate of 0.25 ml min^{-1} was used for the analysis of CYSP. A 100 μl injection volume and a UV detection wavelength of 220 nm was used for the analysis of the compound.

2.5. Evaporated film preparation

A total of 0.5 mg each of β-END and CYSP were weighed into separate 1 ml volumetric flasks and deionized water was added to volume. Solid films were prepared by evaporation of each solution under vacuum onto a calcium fluoride disk. The resultant deposit was examined under the IR microscope to obtain a spectrum of each polypeptide.

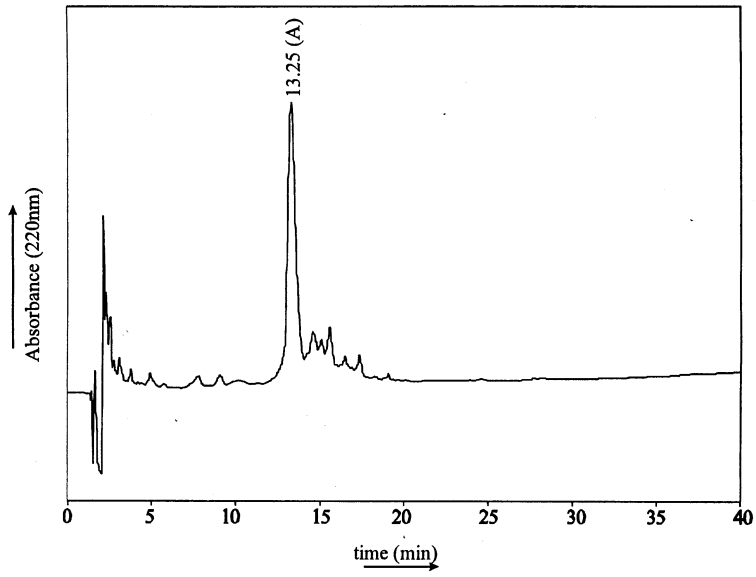

Fig. 3. Typical chromatogram of β endorphin (A) in 0.9% sodium chloride injection USP. For chromatographic conditions, see Section 2.

2.6. Particle beam deposit collection

Deposits of the model polypeptides were collected with the use of the particle beam LC/FT-IR spectrometer (Fig. 1). The deposits were collected onto a calcium fluoride disk and investigated with an IR microscope as previously described by Turula and de Haseth [21,22]. Silica tubing (25 μm i.d.) was used to produce an aerosol and connected the UV detector to the particle beam interface. Care was taken to ensure that the HPLC column back pressure did not exceed 4000 psi. The distance of the calcium fluoride disk from the momentum separator in the interface was optimized at 5 mm to obtain uniform deposits of approximately 50–100 μm in diameter.

2.7. Mass spectral collection

The masses of the polypeptides β-END and CYSP were confirmed by LC-MS. A megaflow electrospray probe was used and the polypeptides were analyzed under the chromatographic conditions described previously. The polypeptides were collected and reconstituted in ammonium acetate buffers of various pH. Ammonium acetate buffers of various pH were also used as the mobile phase carriers and the peptides were flow injected (KD Scientific model 200 syringe pump) into the system. The flow rate was set at 10 μl min^{-1} for both peptides. The needle and cone voltages for the analysis of β-END were 3.44 kV and 36 V, respectively, and 3.31 kV and 32 V, respectively, for CYSP. The MS data were obtained in triplicate and analyzed by plotting the% base peak intensities versus the charge state 'n' of the multiply charges $(M+nH)^{n+}$ ions of peptides at various pH values.

3. Results and discussion

The β endorphin (A) and CYSP (B) eluted at 13.25 and 14.3 min, respectively, under the HPLC gradient elution conditions described above. Typical chromatograms of the two polypeptides are shown in Fig. 3 and Fig. 4 respectively. Each polypeptide was individually collected with the particle beam interface onto a calcium fluoride window and subjected to infrared analysis [35]. The deposit collection was performed using a 25 μm silica tubing and a narrow bore column at a

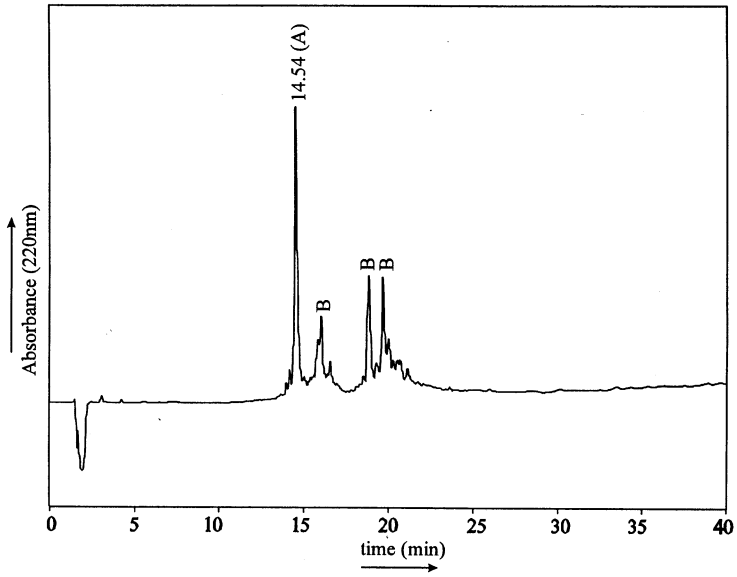

Fig. 4. Typical chromatogram of CYSP (A) and other impurities (B) in water. For chromatographic conditions, see Section 2.

flow rate of 0.25 ml min^{-1}. This disk was examined offline with the use of IR microscopy.

The PB interface can trap and preserve the secondary structure of a peptide, polypeptide or a protein. Experiments previously performed indicated that there was no significant alteration in the structure and function of proteins during PB operation [21,22]. It had also been demonstrated that the deposits collected with the PB technique retained the solution conformation of the protein prior to nebulization. Since the PB operated at sub-ambient temperatures, the biomolecule never attains the activation energy necessary for global unfolding during deposit collection, thereby its solution conformation is preserved. The preservation of biological activity during PB LC/FT-IR spectrometry has also been confirmed with a series of spectrophotometric biological assays reported by Turula and de Haseth [21].

Higher-order protein structural changes have also been studied using ESI/MS with a good correlation between solution state conditions and ion intensity profiles has been established. The effect of various conditions such as pH, temperature and organic solvent content on the conformation of model peptides/polypeptides and proteins have been investigated with ESI/MS. Hydrogen exchange ESI/MS has also been demonstrated as a very efficient technique to study conformational changes in proteins. This has led to the hypothesis that transport of proteins from the solution phase to the gas phase via the electrospray interface maintains higher order protein structure [30]. Recently ESI-MS has been used with great success to monitor the acid-induced folding of proteins [33,34]. Experiments performed previously with Ang I, a decapeptide and POMC-X, an octapeptide indicated that particle beam and electrospray interfaces produced spectra which were very sensitive to solution state conformation [35]. Preliminary results obtained using these model peptides indicated that a combination of these techniques could be useful to study the solution state stability of peptides. However, these peptides were only 8–10 residues long and did not have a definite charge state distribution pattern. Hence, in these studies, larger polypeptides β-END and CYSP, 31 and 21 residues in length, were chosen as model compounds and their conformations were investigated under various solution state conditions.

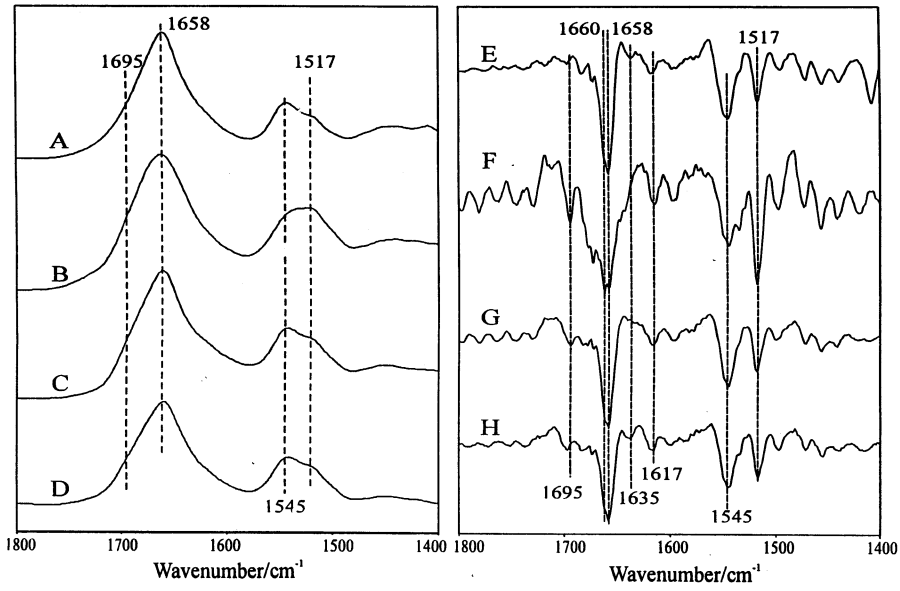

Fig. 5. Infrared (IR) and second derivative spectra of β endorphin from (A,E) particle beam (PB) deposit collected with the column and gradient conditions (for details, see Section 2), (B,F) PB deposit using mobile phase and flow injection, (C,G) PB deposit obtained from water and (D,H) evaporated film.

3.1. Evaporated film, PB analysis of β-END, CYSP

Evaporated films of β-END were prepared by the placement of a drop of a concentrated peptide solution (in water) onto a calcium fluoride disk and its evaporation under vacuum. The evaporated film (Fig. 5D) examined with IR microscopy showed the predominance of a α-helical conformation. This was highlighted by the presence of a strong amide I band at 1655–1658 cm^{-1} (Fig. 5). Mild deconvolution (not shown) and second derivative spectroscopy (Fig. 5) were used to confirm the presence and position of these bands. Apart from the band at 1658 cm^{-1}, there was a strong band at 1660 cm^{-1} which was indicative of the presence of disordered conformation. Weak bands at 1635 and 1695 cm^{-1} indicate the presence of a small percentage of β sheets in the molecule. This was in agreement with previous experiments performed with circular dichroism spectrometry and scanning micro calorimetry that have indicated the conformation of β-END in solution to be predominantly α-helical [36,37]. β-END (0.2 mg ml^{-1}) was flow injected into the particle beam interface and the deposits collected were examined to see if the PB interface could be used in our studies. PB spectra of β-END collected from an evaporated film and after flow injection using 50: 50 (v/v)-0.1% (v/v) aqueous TFA: acetonitrile containing 0.1% TFA (v/v) were examined. This experiment was performed to observe changes, if any, in the conformation of β-END under elution conditions similar to those using a C-18 column. The PB and second derivative spectra (Fig. 5B and 5F, respectively) were once again very similar to the PB spectra obtained from water and the evaporated film, indicating that there was no change in the conformation of β-END under the mobile phase conditions. The column was then introduced into the chromatographic system and a PB deposit of β-END eluting from the column was obtained. The IR spectra of the PB deposit were comparable to the IR spectra obtained using flow injection of β-END. This indicated that the introduction of a column into the system had no significant effect on the conformation of β-END. The high degree of similarity in the IR spectra indicated that PB LC/FT-IR spectrometry did not alter the confor-

mation of the peptide and could be used for the conformational analysis of β-END in solution.

The evaporated film of CYSP indicated that its conformation was totally different from that of β-END. The IR and second derivative spectra (spectra not shown) of CYSP had a very strong band at 1622–1628 cm^{-1} which confirmed the presence of a strong β sheet conformation. A band at 1695 cm^{-1}, seen as a broad envelope in the IR spectra but as a clear strong signal in the second derivative spectra, was indicative of β-bends and confirmed the presence of β-sheets in the molecule. There was a weak band at 1662 cm^{-1} that was indicative of the presence of a disordered structure in the molecule. There was also a weak band at 1680 cm^{-1} which was further indicative of β-bends in the molecule. Amide II bands at 1550 cm^{-1} and at 1522 cm^{-1} were also seen in the IR spectra. PB deposits collected using flow injection of the mobile phase also produced spectra that were comparable to the evaporated film indicating that the mobile phase did not alter the conformation of CYSP. PB deposits collected after elution of CYSP from an octadecylsilane column were similar to those obtained using flow injection (without a column), thereby indicating that the method developed could be used for the conformational analysis of CYSP in solution.

3.2. PB LC/FT-IR analysis of β-END and CYSP in parenteral solutions

The β-END and CYSP were then reconstituted in 0.9% sodium chloride injection USP, D5W USP and sterile water for injection USP and the conformation of the model polypeptides was examined over a 24-h period (Figs. 6 and 7). HPLC effectively separated the peptides from any impurities formed over the 24-h period and PB LC/FT-IR was used to monitor the conformational changes of each polypeptide. Particle beam spectra of β-END from sterile water for injection USP, 0.9% sodium chloride injection USP and 5% dextrose injection USP (D5W) at 0 h and after a 24-h storage time are presented in Fig. 6. There was a strong band at 1658 cm^{-1} in all spectra which indicated the presence of an α-helical conformation. The presence of a weak band at 1664 cm^{-1} indicated the presence of disordered conformation in the molecule. Mild deconvolution (not shown) and second derivative spectroscopy were used to identify the position of certain bands. Weak bands at 1695 and 1625–1630 cm^{-1} indicated the presence of a small percentage of β-sheet in the molecule. Amide II bands were also observed at 1545 and 1520 cm^{-1}. The intensity and the position of the bands were similar in all the parenteral carriers indicating that irrespective of the type of carrier used to reconstitute the compound, β-END had the same conformation. When deposits of β-END stored in the various parenteral mixtures over 24 h at ambient temperature (23 ± 2°C) were examined, it was observed

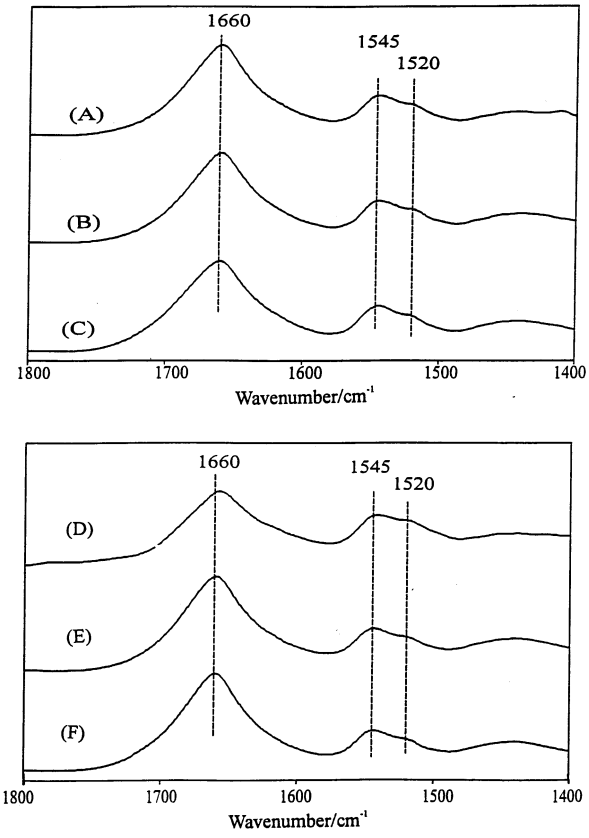

Fig. 6. Particle beam deposits of β endorphin from (A) sterile water for injection USP, (B) 5% dextrose USP and (C) 0.9% sodium chloride solution USP at 0 h and (D) sterile water for injection USP, (E) 5% dextrose USP and (F) 0.9% sodium chloride solution USP at 24 h.

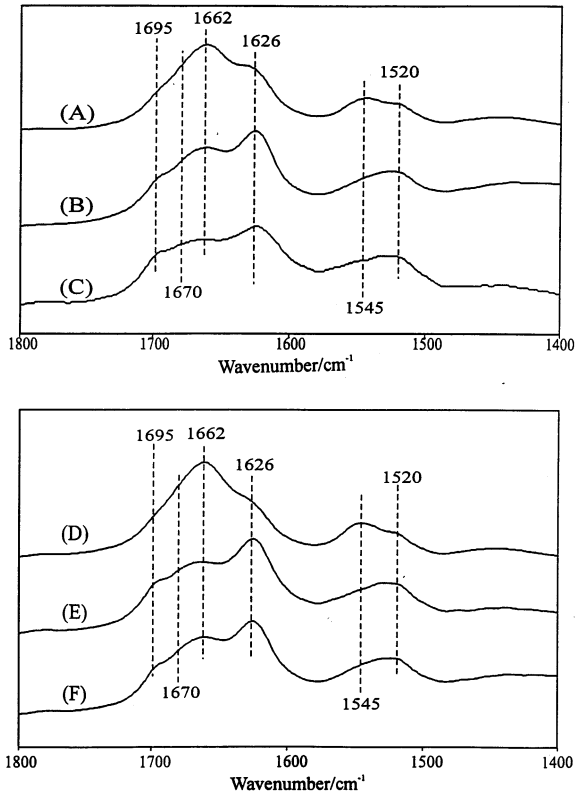

Fig. 7. Infrared spectra of CYSP at 0 and 24 h, respectively from (A,D) 5% dextrose USP, (B,E) 0.9% sodium chloride solution USP and (C,F) sterile water for injection USP.

that there was practically no change in the conformation from that seen at 0 h indicating that β-END was very stable when stored for 24 h at ambient temperature.

When polypeptide deposits collected from the parenteral solutions were examined, it was observed that the IR spectra of CYSP deposits from sterile water for injection (Fig. 7A) and 0.9% sodium chloride injection (Fig. 7B) were similar. The spectra contained strong bands at 1622–1628 cm^{-1} which were representative of a β-sheet conformation and the weak band at 1662–1664 cm^{-1} represented disordered conformation. There were also amide I bands at 1695 cm^{-1} and at 1680 cm^{-1} representative of β-bends. There was a weak band at 1655–1663 cm^{-1} indicative of a slight α-helical character in the molecule. The relative intensity of the β-sheet band was slightly greater in 0.9% sodium chloride injection USP than in sterile water for injection USP. The IR spectrum of CYSP deposits obtained from D5W (Fig. 7C) was slightly different in that the primary bands were that of a disordered conformation and were observed at 1662–1666 cm^{-1}. The disordered bands were much more intense than the β-sheet bands at 1628–1630 cm^{-1}. There were weak IR bands seen at 1695 cm^{-1} and at 1680 cm^{-1} indicative of β-bends present in the molecule. This difference in the relative intensity of the β-sheet and bands in D5W was attributed to a change in conformation. The solutions were stored at ambient temperature (23 ± 2°C) for 24 h and their PB deposits were collected and examined. The IR spectra of CYSP obtained after a 24 h storage period in D5W at ambient temperature (Fig. 7D) indicated that there was no change in the molecule over time. The primary IR bands were still disordered and were observed at 1662–1666 cm^{-1} while β-sheet bands were observed at 1624–1630 cm^{-1}. The intensity of the disordered bands was still greater than the intensity of the β-sheet bands. The IR spectra of PB deposits of CYSP collected after a 24 h storage time in sterile water for injection USP and 0.9% sodium chloride injection USP were also similar to the deposits collected at 0 h. The primary IR band representing a β-sheet was observed at 1622–1628 cm^{-1} while there was a weak band at 1662–1664 cm^{-1} which represented a disordered conformation. The spectra was identical both in intensity and in the position of bands to that obtained at 0 h indicating that the molecule had not undergone a change over time. The CYSP had retained its conformation in all three parenteral carriers when stored 24 h at ambient temperature (23 ± 2°C). Whether the difference in conformation observed in D5W as compared to sterile water for injection USP or 0.9% sodium chloride injection USP would lead to a change in the biological activity of CYSP is unknown.

3.3. Effect of pH on β-END and CYSP conformation

Previous studies from this laboratory using various buffers on the PB apparatus have indicated

that ammonium acetate buffer was the most compatible buffer primarily due to its volatility [38]. Furthermore, the interference produced by ammonium acetate in the IR spectra of the peptides was also minimal. Ammonium acetate buffers of various pH were used to reconstitute the peptides and PB deposits were collected. The IR bands of β-END indicated the presence of a small percentage of α-helical conformation under all conditions with a band at around 1655–1660 cm^{-1} (Fig. 8). The second derivative spectra of β-END indicated the presence of a slight amount of disordered conformation. As with parenteral mixtures, there were weak bands at 1625–1630 cm^{-1} and 1695 cm^{-1} that indicated the presence of β-sheets in the molecule. As the pH increased from 2–10, there was little change observed in the conformation of the molecule. The relative intensity and position of the various IR bands were the same, thereby indicating that there was little, if any conformational change in the molecule with a change in the pH of the solution.

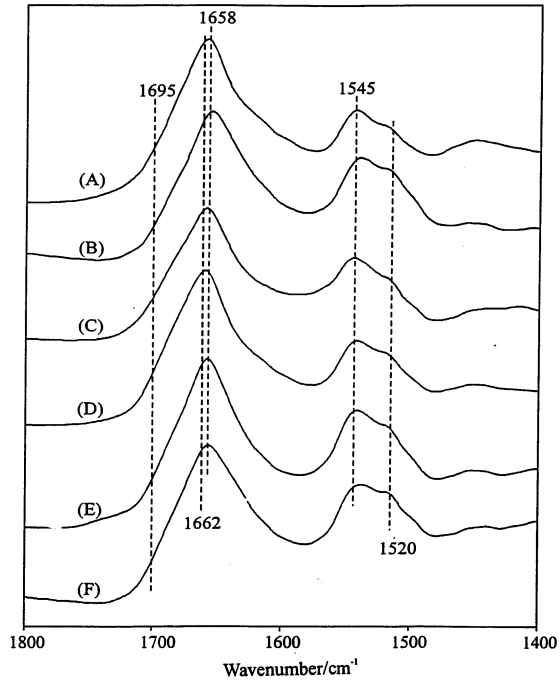

Fig. 8. β Endorphin: infrared spectra of particle beam deposits from (A) pH 2.0, (B) pH 4.0, (C) pH 6.0, (D) water (E) pH 8.0 and (F) pH 10.0.

It is well known that peptides and proteins are very susceptible to denaturation at extremes of pH. Denaturation leads to unfolding of the biomolecules which result in a change in the ESI charge state intensities. As the peptides or proteins unfold, the number of exposed amino acids increases thereby increasing the number of ionizable sites in the molecule. This leads to a shift in the charge state intensities towards lower M/Z values. There have been a number of studies where electrospray ionization (ESI) has been used to study the denaturation of proteins [27–30]. The ESI has been used to probe conformational changes that cause an enzyme to loose activity but cause little variation in UV, CD spectrum and fluorescence intensity [39]. Hence, experiments were performed to see if the results obtained using ESI-MS correlate to those obtained using PB spectrometry. The charge state distributions of β-END obtained at a pH range of 2–10 are shown in Fig. 9. It was observed that a change in the pH did not result in a change in the charge state distribution of β-END indicating that the molecule was extremely stable to variations in pH. This correlated well with the IR data obtained using the PB technique. Hence, it could be concluded that β-END was stable in a pH range of 2–10 and maintained its α-helical character.

The PB deposits of CYSP were collected at various pH values (Fig. 10) Examination of the solutions indicated a slight change in the conformation of CYSP with a change in the pH. It was observed that the primary peak in all the deposits was around 1624–1630 cm^{-1} which was indicative of a β-sheet conformation. This was further confirmed by the presence of strong IR bands observed at 1695–1700 cm^{-1}. These bands were characteristic of β-bends. Apart from this, strong IR bands at 1665–1670 cm^{-1} and weak bands at 1656–1663 cm^{-1} representative of disordered conformation and α-helical conformation were also observed. However, the intensity and position of these bands varied slightly with a change in pH. The IR (Fig. 10D) and second derivative spectra for water indicated that CYSP in water had IR bands representing a β-sheet, disordered conformation and a slight percentage of α-helical character. As the pH decreased from 7 to 2, it was

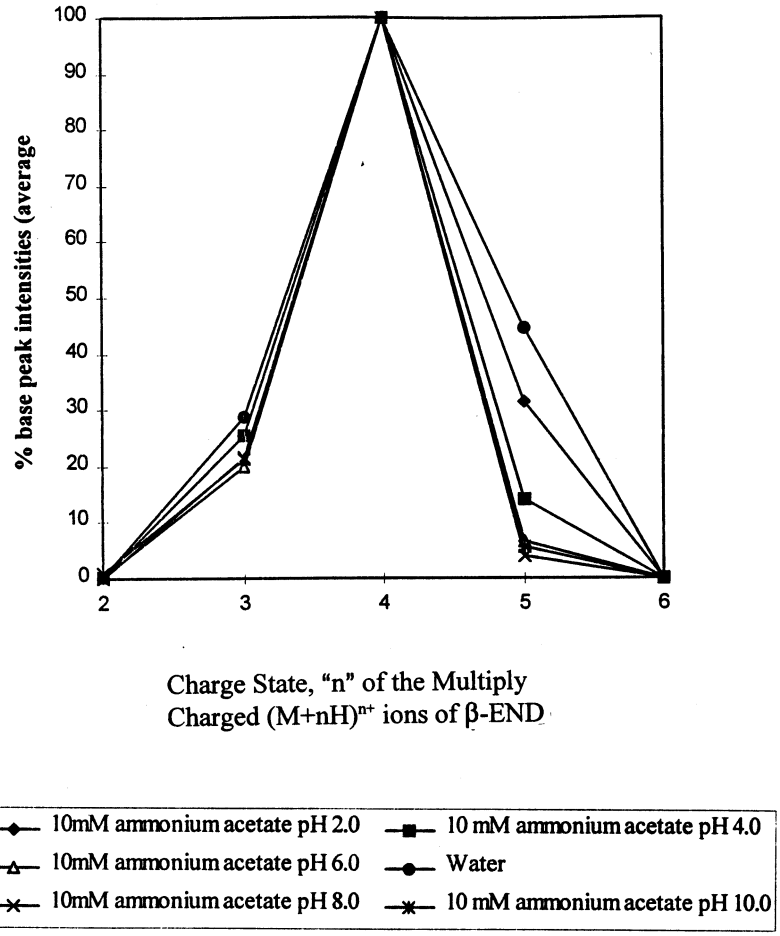

Fig. 9. Effect of pH on the charge state intensities of β endorphin.

observed that the relative intensity of the disordered band relative to the β-sheet band increased thereby, indicating a change in the conformation of the molecule with a decrease in pH. As the pH increased from 7 to 8, there was little change seen in intensity or position of the bands, However as the pH increased to 10.0, there was an increase in the intensity of the band at 1665 cm^{-1} (disordered conformation) relative to the β-sheet band. This data was interpreted under the assumption that the population equilibrium at a pH of 7.0 (water) was representative of the native conformation of CYSP. Similar studies were carried out using the electrospray MS and the charge state intensities were observed as a function of a change in pH (Fig. 11). The CYSP has 5 charge states (3–7) and it was observed that the ion with a $+5$ charge state in water was most intense (Fig. 11). At all other pH's, the ion with a $+4$ charge state was most intense while the ion with a $+5$ charge state had a relative intensity of 60–70%. This indicated that there was a slight change in the conformation of the molecule with a change in pH. It was also observed that as the pH increased to 10.0 or decreased to 2, the charge state intensity profile shifted more towards lower M/Z values. This shift in the charge state intensity (envelope) profile with a change in pH was interpreted as a slight change in the conformation of the molecule and was consistent with the IR data. The observed shift in the charge-state distribution could also be caused by the addition or removal

of charge sites as the pH of the solution approaches or exceeds the pK_a value of acidic and basic side-chains. These changes in side-chain charging may well be the cause of the observed conformational changes rather than the conformational changes exposing additional charge sites.

3.4. Effect of temperature on β-END and CYSP conformation

The effect of temperature on the conformation of the model polypeptides was also examined (Figs. 12 and 13). PB deposits of β-END heated to temperatures of 50, 75 and 100°C were collected and examined under the IR microscope. The IR spectra (Fig. 12) collected from β-END were similar to the IR spectrum obtained from water at ambient temperature. The primary band was observed at 1655–1662 cm^{-1} and represented a α-helical conformation. Apart from this, there were IR bands characteristic of disordered conformation and β-turns. There was no difference in the IR spectra collected at various temperatures indicating that the conformation of the molecule was very stable to a change in temperature.

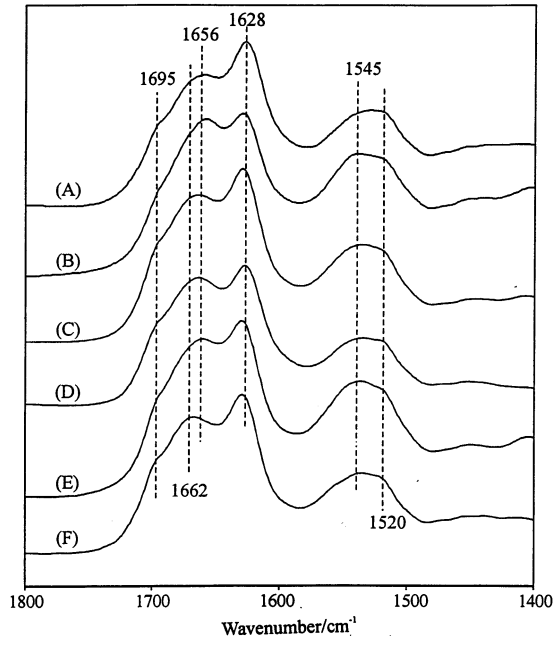

Fig. 10. CYSP: Infrared spectra of particle beam deposits from (A) pH 2.0, (B) pH 4.0, (C) pH 6.0, (D) water (E) pH 8.0 and (F) pH 10.0.

The IR spectra of CYSP deposits heated to temperatures of 50, 75 and 100°C (Fig. 13) were similar to IR spectra of PB deposits collected from water at ambient temperature. The IR band at 1625–1630 cm^{-1} indicative of a β-sheet was observed in all the spectra. The IR spectra collected from a solution heated to 75°C when compared to a spectra collected from solutions stored at ambient temperature or heated to 50°C indicated that there was an increase in the intensity of the disordered conformation band with an increase in the temperature of the solution. This indicated a change in the conformation of the molecule with an increase in temperature. IR spectra collected from PB deposits of a solution heated to 100°C indicated that the intensity of the disordered conformation band relative to the β-sheet band had increased considerably, thereby, indicating a further change in the conformation of the molecule. This increase in the intensity of the β-sheet band was assumed to be due to intermolecular hydrogen bonding between the denatured molecules.

4. Conclusions

The use of PB LC/FT-IR as a tool for the investigation of conformational changes in polypeptides such as β-END and CYSP has been described. The β-END had an α-helical conformation and was very stable to a change in environmental conditions. The conformation of the molecule in the various parenteral carriers was identical thereby indicating that the molecule was very stable in all the carriers tested. CYSP had a β-sheet conformation and was sensitive to the parenteral carriers. PB deposits of CYSP in D5W when compared to PB deposits from water or 0.9% sodium chloride injection showed a difference. The conformation

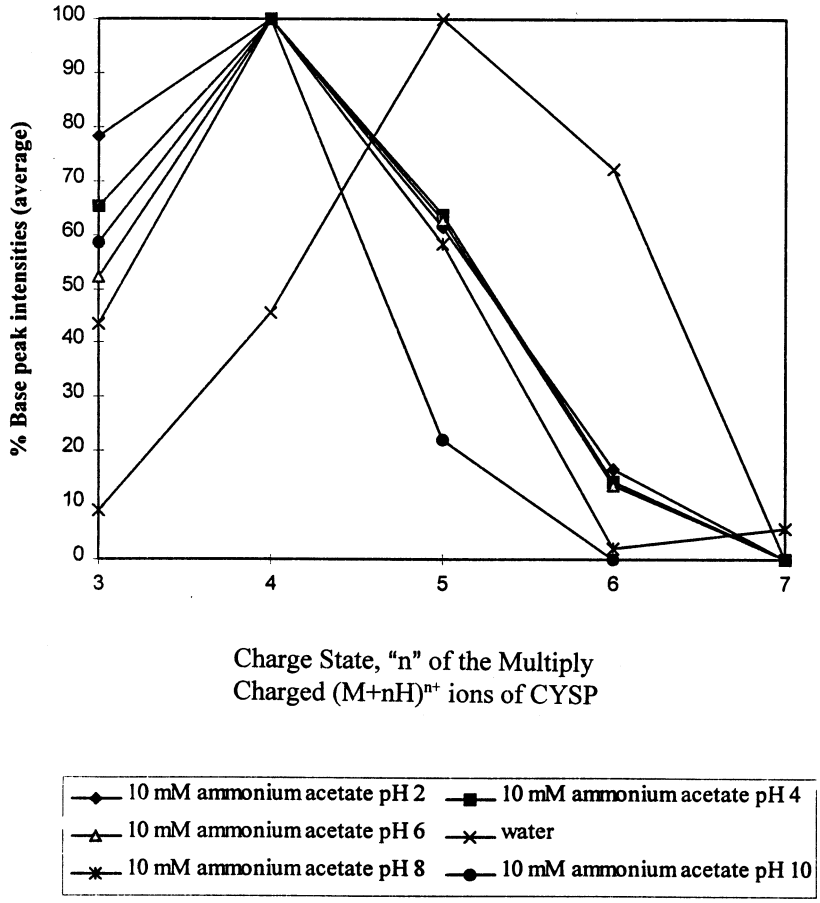

Fig. 11. Effect of pH on the charge state intensities of CYSP.

of β-END and CYSP did not change over a 24 h period indicating that the molecule was conformationally stable. CYSP reconstituted in D5W retained its disordered conformation thereby indicating that it was conformationally stable over a 24 h period. A change in the pH from 2–10 did not affect the conformation of β-END, but there was a change in the conformation of CYSP. As the pH of the solution decreased from 7 to 2, the intensity of the disordered conformation band in CYSP increased and this was accompanied by a corresponding decrease in the β-sheet intensity of the molecule. As the pH increased from 7–10, a change similar to that observed with decreasing pH was noticed. This indicated that CYSP was sensitive to a change in pH and undergoes conformational changes at extremes of pH. This observation was further confirmed using electrospray MS where a change in the charge state intensities with a change in pH was observed. Temperature studies with β-END indicated no change in the molecule with an increase in temperature. However, temperature studies with CYSP indicated a conformational change at higher temperatures. Hence, from these experiments, it can be concluded that the technique can be used to study polypeptides that are 20–35 residues long. Presently the technique is being extended to study proteins that have a much longer chain length.

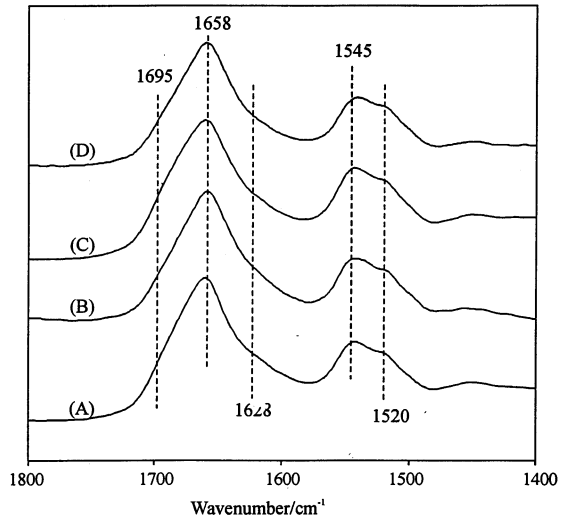

Fig. 12. Effect of temperature on the conformation of β endorphin, (A) ambient (23°C), (B) 50°C, (C)75°C and (D) 100°C.

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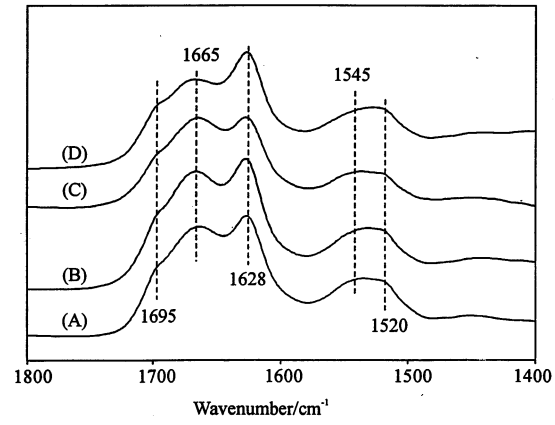

Fig. 13. Effect of temperature on the conformation of CYSP, (A) ambient (23°C), (B) 50°C, (C) 75°C and (D) 100°C.

References

[1] C.J. Wordell, Am. Drug. 202 (1990) 18–26.
[2] W.S. Hancock, B. Willis, Am. Lab. June (1996) 31–34.
[3] M.C. Manning, K. Patel, R.T. Borchardt, Pharm. Res. 6 (11) (1989) 903–918.
[4] T. Chen, Drug Dev. Ind. Pharm. 18 (11, 12) (1992) 1311–1354.
[5] J.S. Andersen, B. Svensson, P. Roepstoff, Nat. Biotechnol. 14 (1996) 449–457.
[6] M.I. Aguilar, M.T.W. Hearn, in: M.T.W. Hearn (Ed.), HPLC of Proteins, Peptides and Polynucleotides, VCH, New York, 1991, pp. 247–275.
[7] P. Oroszlan, R. Blanco, X.M. Xu, D. Yarmush, B.L. Karger, J. Chromatogr. 500 (1990) 481–502.
[8] G.K. Sofer, Biotechnology 4 (1986) 712.
[9] D.R. Nau, in: M.T.W. Hearn (Ed.), HPLC of Proteins, Peptides and Polynucleotides, VCH, New York, 1991, pp. 333–393.
[10] P.M. Young, R.F. Burgoyne, T.E. Wheat, Pept. Res. 3 (2) (1990) 81–84.
[11] M. Kai, Y. Ohkwia, Trends Anal. Chem. 6157 (1987) 116–120.
[12] R. Newcomb, LC-GC 10 (1) (1992) 34–39.
[13] J.A. Loo, C.G. Edmonds, R.D. Smith, Science 248 (1990) 201–204.
[14] K.L. Biemann, H.A. Scoble, Science 237 (1987) 992–998.
[15] E.C. Huang, J.D. Henion, J. Am. Soc. Mass Spectrom. 1 (1990) 158–165.
[16] A. Elliot, E.J. Ambrose, Nature 165 (1950) 921–922.
[17] H. Susi, in: S.N. Timasheff, G.D. Fasman (Eds.), Structure and Stability of Biological Molecules, Marcell Dekker, New York, 1969, pp. 575–633.
[18] P.R. Griffiths, A.R. Lange, J. Chromatogr. Sci. 30 (1992) 93–97.
[19] S.L. Jordan, J. Taylor, J. Chromatogr. Sci. 35 (1997) 7–13.
[20] J.L. Dwyer, A.E. Chapman, X. Liu, LC-GC 13 (3) (1995) 244–248.
[21] V.E. Turula, J.A. de Haseth, Appl. Spectrosc. 48 (1994) 1255–1264.
[22] J.A. de Haseth, V.E. Turula, Mikrochim. Acta Suppl. 14 (1997) 109–119.
[23] R.T. Bishop, V.E. Turula, J.A. de Haseth, R. Ricker, in: D.R. Marshak (Ed.), Techniques in Protein Chemistry—VIII, Academic Press, San Diego, 1997, pp. 165–176.
[24] V.E. Turula, R.T. Bishop, R. Ricker, J.A. de Haseth, J. Chromatogr. A. 763 (1997) 91–103.
[25] V.E. Turula, J.A. de Haseth, Anal. Chem. 68 (1996) 629–638.
[26] R.T. Bishop, V.E. Turula, J.A. de Haseth, Anal. Chem. 68 (1996) 4006–4014.
[27] Y. Liu, D.L. Smith, J. Am. Soc. Mass Spectrom. 5 (1994) 19–28.
[28] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 6471.
[29] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga, H.R. Udseth, Anal. Chem. 62 (1990) 882–899.

[30] S.K. Chowdhury, V. Katta, B.T. Chait, J. Am. Chem. Soc. 112 (1990) 9012–9013.
[31] C.L. Stevenson, R.J. Anderegg, R.T. Borchardt, J. Am. Soc. Mass Spectrom. 4 (1993) 646–651.
[32] R.T. Bishop, J.A. deHaseth, Microchim Acta Suppl. 14 (1997) 721–724.
[33] L. Konermann, D.J. Douglas, Biochemistry 36 (1997) 12296–12302.
[34] L. Konermann, F.I. Rosell, A.G. Mauk, D.J. Douglas, Biochemistry 36 (1997) 6448–6454.
[35] T.G. Venkateshwaran, J.T. Stewart, R.T. Bishop, J.A. de Haseth, M.G. Bartlett, J. Pharm. Biomed. Anal. 17 (1998) 57–67.
[36] A.A. Makarov, V.M. Lobachov, I.A. Adzhubei, N.G. Esipova, FEBS Lett. 306 (1) (1992) 63–65.
[37] V.M. Lobachev, A.A. Makarov, I.A. Adzhubei, N.G. Esipova, Biophysics 37 (5) (1992) 757–763.
[38] R.B. Robertson, J.A. de Haseth, R.F. Browner, Appl. Spectrosc. 44 (1) (1990) 8–13.
[39] X.M. Pan, X.R. Sheng, S.M. Yang, J.M. Zhou, FEBS Lett. 402 (1997) 25–27.