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RAPID CHARACTERIZATION OF PEPTIDE SECONDARY STRUCTURE BY FT-IR SPECTROSCOPY

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Use of peptides and proteins in many different applications requires a more detailed structural characterization of these in different environments. Information on structural properties of peptides and proteins in various solvents are also given by Fourier transform infrared (FT-IR) spectroscopy. Here, we investigate the amide I, II, and III bands of peptides using both FT-IR spectra and their second derivatives. This paper reveals a large body of information obtained by FT-IR technique that can be used to understand the structure and stability of some peptides and proteins in different environments. Circular dichroism (CD) spectroscopy support data obtained within FT-IR experiments.

INTRODUCTION

FT-IR spectroscopy has recently become very popular for structural characterisation of proteins.[1,2] Difference spectroscopy has the advantage in providing highly detailed information on conformational changes in proteins.[3] Infrared spectroscopy is being increasingly utilized for the analysis of peptides and proteins because it probes the universally available amide (peptide) bonds, which display distinct IR signals for differently folded peptides and proteins.[4] FT-IR spectra of small peptides can be highly complex and extremely difficult to interpret. Amide bands associated with different secondary structural elements in peptides can show large deviations from those observed with large water-soluble proteins. This is partly due to the fact that small peptides are often highly flexible and are solvated to a greater extent compared to globular proteins. Peptide aggregation can also result in the appearance of additional bands in the amide I region that can be erroneously attributed to peptide structure. These are amongst a host of factors that need to be taken into consideration when interpreting infrared spectra of peptides.[5] The polypeptide and protein repeat units give rise to nine characteristic IR absorption bands, namely, amide A, B, and I−VII. Of these, the amide I and II bands are the two most prominent vibrational bands of the protein backbone.[6] Banker described the nine amide vibration modes and some standard conformations in detail in his review.[7] Amide I (1600−1690 cm^{-1}, C=O stretching), amide II (1480−1575 cm^{-1}, CN stretching, NH bending), and amide III (1229−1301 cm^{-1}, CN stretching, NH bending) are the most used IR bands to reveal the conformational changes of the proteins and peptides.

FT-IR spectra can be obtained for proteins and peptides in a wide range of environments and direct correlations between the IR amide I band frequencies and the secondary structure components can be found.[8] The purpose of this article is to show the applications of FT-IR [9-11] technique to the study of protein secondary structure and conformational changes of some newly synthesized peptides.[12,13] The results were compared to those from protein investigations as recommended by Bagińska *et al.*[14] CD spectroscopy spectra and the FT-IR ones bring rather similar information concerning the peptide secondary structure. Finally, several new approaches

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of FT-IR spectroscopy are discussed and the biological applications of this technique are mentioned.[10]

MATERIALS AND METHOD

Materials

All chemicals were of analytical reagent grade and were purchased from Fluka, Aldrich or Merck. We used here ovalbumin (Alb) as model protein (from Sigma-Aldrich Chemie, Deisenhofen, Germany), Aβ1-40 peptide (Aβ), which was provided by GenicBio (BioTech Co., Shanghai, China), and two newly synthesized 19-amino acid residue peptides.

The peptides H_2N-GGGGHGGGGHGGGGHG GGG-COOH (P1) and H_2N-GGGHGGGHGGG HGGGGGGG-COOH (P2) (where G is glycine and H is histidine) were automated synthesized with a peptide synthesizer (Research Scale Peptide Synthesis from INTAVIS Bioanalytical Instruments AG, Köln, Germany) on Fmoc-Gly-Wang resin. A pre-defined protocol and ready-to-use amino acid cartridges of the synthesizer were used. The final result was a support carrying the desired peptide of choice which was then cleaved off manually.

Sample preparation

Peptide and protein solutions were prepared with redistilled water. Gly-peptides P1 and P2 peptides were dissolved in hexafluoropropanol: water (molar ratio 1:1) solvent. Then, the 1 mg mL^{-1} protein solutions were evaporated to dryness on a glass plate in fresh air, followed by a complete drying in a dessicator with calcium chloride.

Instruments

FT-IR spectra were recorded on a Shimadzu Model 8400S FT-IR spectrophotometer.

CD spectra were recorded using a Jasco-715 Spectropolarimeter (Labor and Datentechnik GmbH, Germany).

FT-IR spectroscopy

The samples (1-2 mg) were mixed with KBr, pulverized, and formed into a disk-shaped pellet. All spectra were recorded in the frequency region 500 – 4000 cm^{-1}, under a resolution of 2 cm^{-1} and with a scanning speed of 2 mm sec^{-1} and 20 scans per sample. The spectral processing and the determination of the secondary structures from the intensities of bands in the second derivative amide I spectra were performed as described by Caughey *et al*.[11]

CD spectroscopy

The measurements were performed in quartz cells with a path length of 0.5 mm, in the range from 180 to 260 nm. Six individual data were averaged by device in order to obtain the reported CD spectrum. The measurements were carried out at room temperature. Proportions of each secondary structure type are obtained by spectral deconvolution of the peptide CD spectrum as a linear sum of predetermined basis spectra.[12,13] In addition, we deconvoluted CD spectra as shown by Perez-Iratxeta and Andrade-Navarro.[15] The K2D2 server is publicly accessible at http://www.ogic.ca/projects/k2d2/.

Data processing

We analyzed the whole wavenumber range from 500 to 4000 cm^{-1}, and focused mainly on the representative regions of wavenumbers, such as amide I and amide II regions and the fingerprint area. In addition, we calculated the second derivatives of FT-IR spectra to show the most important peaks of absorption. Then, we compared the information concerning peptide conformations obtained by FT-IR spectroscopy with that from CD technique.

RESULTS AND DISCUSSION

FT-IR is one of the few techniques that can be applied for structural characterization of peptides and proteins in different environments. The amide I band – between 1600 and 1700 cm^{-1} – was the most intense absorbance band for all the investigated proteins and peptides, being mainly associated with the C=O stretching vibration and directly related to the backbone conformation. This band had a characteristic shape for each peptide investigated. We compared here the absorbance of proteins (ovalbumin) with that of Aβ1-40 peptide (the peptide involved in Alzheimer disease) and two newly synthesized smaller peptides (P1 and P2) to reveal their significant similarities or differences.

FT-IR spectra of proteins

Amide I and amide II bands are two major bands of the protein infrared spectrum and are conformationally sensitive. Amide II results mostly from the N—H bending vibration and from the C—N stretching vibration (18-40%). Important bands associated with various peptide conformations were also found below 1,400 cm^{-1} (amide III). Generally, the 1,655 cm^{-1} peak, which is generally assigned to α-helix conformation, was positively correlated with bands at 1,175; 1,305; 2,950 and 3,330 cm^{-1}, as shown by some other authors.[16-18]

The high absorption of ovalbumin at 1,654 cm^{-1} was correlated with the presence of α-helical conformers in the solid state (Fig. 1). This absorption had the corresponding band in the amide II region at 1,545 cm^{-1}. Moreover, the peak at 1,308 cm^{-1} also indicated the presence of α-helical structures (Table 1). The band observed at 1,670 cm^{-1} was assigned to β-turns. No antiparallel β-sheet (1,698 cm^{-1}) or aggregated strands (1,604 cm^{-1} or 1,611 cm^{-1}) were found.

Aβ1-40 had an intense absorption at 1628 cm^{-1}, corresponding to its high content of β-sheet conformers, which is concordant to amide II band absorptions around 1,530 cm^{-1} at 1,526 cm^{-1}. The 1,662 cm^{-1} signal was assigned to β-turn conformers.

P1 displayed a large band in the range from 1,632 cm^{-1} to 1,680 cm^{-1}, with some maxima of absorption at 1,648 cm^{-1} (random coil), 1,656 cm^{-1} (α-helix), and 1,670 cm^{-1} (β-turn, the most intense signal). The 1,656 cm^{-1} and 1,670 cm^{-1} bands were correlated with important amide II bands at 1,541-1,545 cm^{-1} and 1,528 cm^{-1} (Figs. 1 and 2). In the amide III region, the 1,338 cm^{-1} and 1,242 cm^{-1} bands confirmed the presence of α-helix and β-turn conformers.

P2 showed three peaks in the band area, at 1,632 cm^{-1}, 1,670 cm^{-1}, and 1,697 cm^{-1}, respectively. The Amide I band was larger than that of P1, in the range from 1628 cm^{-1} to 1683 cm^{-1}. The high absorption of P2 peptide at 1,632 cm^{-1} was correlated with the presence of β-sheet conformers in the solid state. The peak from 1,670 cm^{-1} indicated the β-turn structures and the important absorption peak found at 1,697 cm^{-1} is characteristic to antiparallel β-sheet aggregated strands. All these contributions had the corresponding absorption in the amide II region. The main peak was found around 1,518 cm^{-1}. Indeed, the secondary structures of the two peptides, P1 and P2, proved to be different, as shown in Fig. 1.

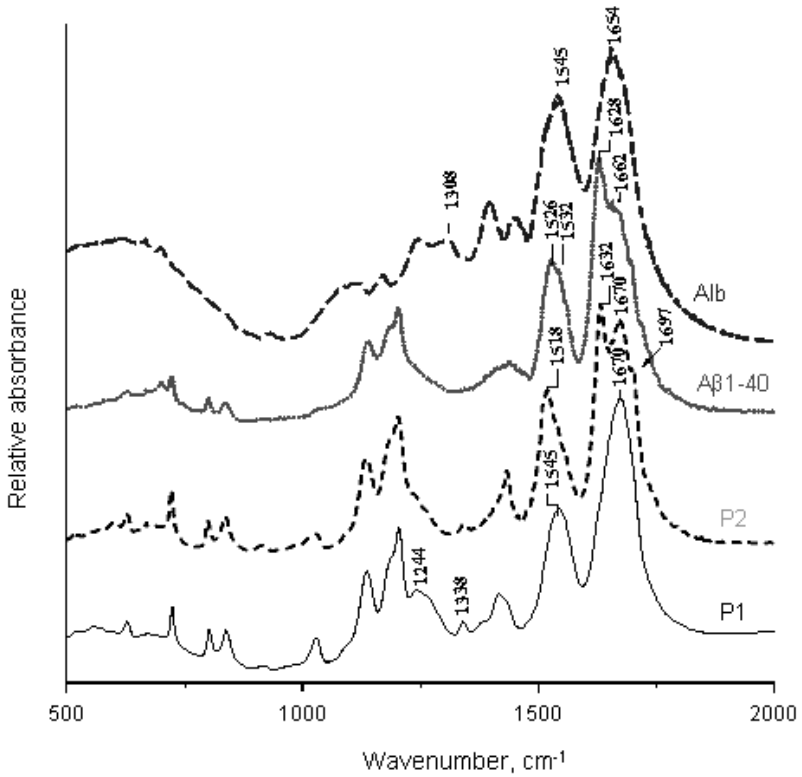

Fig. 1 – FTIR spectra (500-2000 cm^{-1}) of P1 and P2 peptides as well as those of amyloid-β peptide (Aβ1-40) and ovalbumin (Alb).

Table 1

Assignment of amide I-III band positions to secondary structures based on experimental data[18]

Conformation	The position of bands and peaks within amide I-III region
	Amide I band
α-helix	1649; 1653-1657; 1655
β-sheet	1621-1623; 1630; 1634-1639; 1647-1648; 1680-1691 (β-sheet or β-turn)
β-turn	1661; 1667; 1673; 1677
random coil	1648; 1654; 1642-1657
aggregated protein	1615-1620
	Amide II band
α-helix	1545
β-sheet	1530
β-turn	1528; 1577
	Amide III band
α-helix	1293; 1300-1311; 1316-1320; 1331
β-sheet	1223-1225; 1231-1238; 1242; 1248-1251
unordered + turn	1244; 1259-1269; 1280-1290

Second derivative spectra

The second derivative of FT-IR spectra confirmed the differences between the secondary structures of the peptides and proteins investigated here by FT-IR as shown in Fig. 2. In addition, the second derivative spectra clearly resolved peaks, which can be associated with the α-helix, β-strands, and turns. Tentative assignments are proposed, and the observed peaks are related to the secondary structure of the proteins studied. Thus, the signals were more evident than those of FT-IR spectra and could be assigned to various conformations of the investigated polypeptides. For example, the data appear to present a direct spectroscopic evidence of α-helical or turns in a native protein.

Although the broad IR absorption band of peptide P1 had a maximum at 1670 cm^{-1}, characteristic of β-turn conformation, the derivatives of FT-IR spectrum suggest a very different secondary structure of this peptide (Fig. 3). Thus, the second derivative had a strong signal in the negative region at 1648 cm^{-1} attributable to a high content of unordered conformers. The peak at 1655 cm^{-1} was clearly assigned to α-helix, confirming thus the conformation of P1, as seen in Table 2. The high content of unstructured conformers was predictable because of the large number of glycine residues, a non-chiral amino acid, which gives high flexibility to peptide chain of P1.

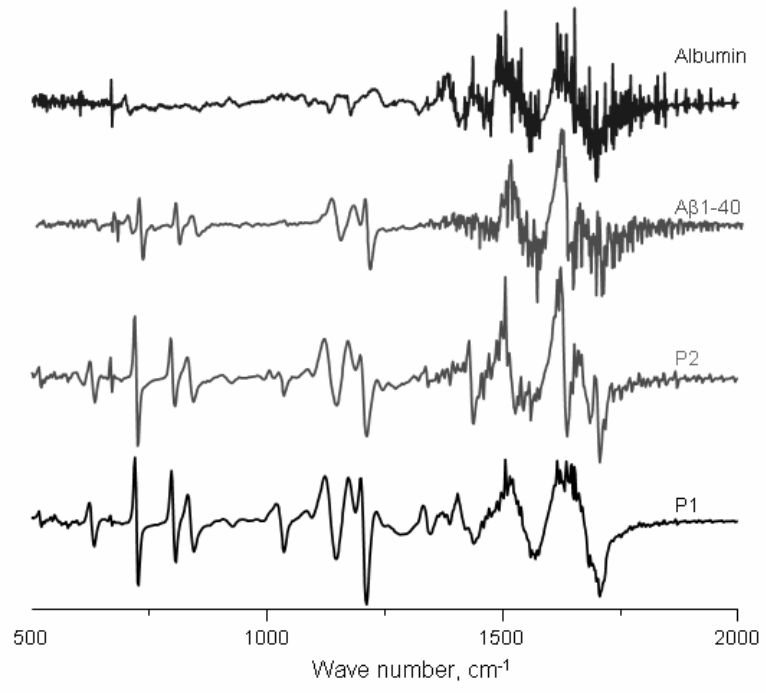

Fig. 2 – Second derivative spectra of P1 and P2 peptides as well as those of amyloid-β peptide (Aβ1-40) and ovalbumin (Alb).

The FT-IR spectrum of peptide P2 contains three maxima in the Amide I band at 1631 cm^{-1} (β-sheet), 1655 cm^{-1} (α-helix), and 1676 cm^{-1} (β-turn), respectively. The band at 1997 cm^{-1} could be assigned either to β-sheet or β-turn populations. CD spectroscopy indicated no β-sheet strctures and, therefore, we assigned this signal to β-turn conformers. Maybe, P2 contains a low proportion of β-sheet conformers, while CD spectroscopy indicated no such structures but a high proportion of α-helix. Possibly, the conformation of P2 is quickly changing in the investigated solutions by CD, whereas FT-IR spectroscopy in solid may afford better prediction for its secondary structure. First order derivative of Amide I band contains much more information than FT-IR spectrum itself, which is observable at 1615 cm^{-1}, 1623 cm^{-1}, 1637 cm^{-1} (negative value), 1645 cm^{-1}, 1653 cm^{-1}, 1669 cm^{-1}, and 1696 cm^{-1}, respectively. These peaks indicated a complex secondary structures (both α-helix, and β-sheet or β-turn). To find the maximum of this band, the second-order derivative of Amide I band should be used. The most intense peak was found at 1630 cm^{-1}, followed by one at 1655 cm^{-1}, indicating a large proportion of β-sheet conformers alongside with α- helical conformers. In fact, the second derivative also indicated the presence of an unordered population (1648 cm^{-1}), a β-turn structure (1670 cm^{-1}), and peptide oligomers (1618 cm^{-1} and 1683 cm^{-1}).

We explained all these conformations by the presence of glycine in the sequence of P2 peptide. Thus, some peptide molecules may contain a helical area, alongside with an unordered one, while others can be folded, with the formation of hydrogen bonds between the two chains. FT-IR spectra may thus display an average conformation of individual molecules.

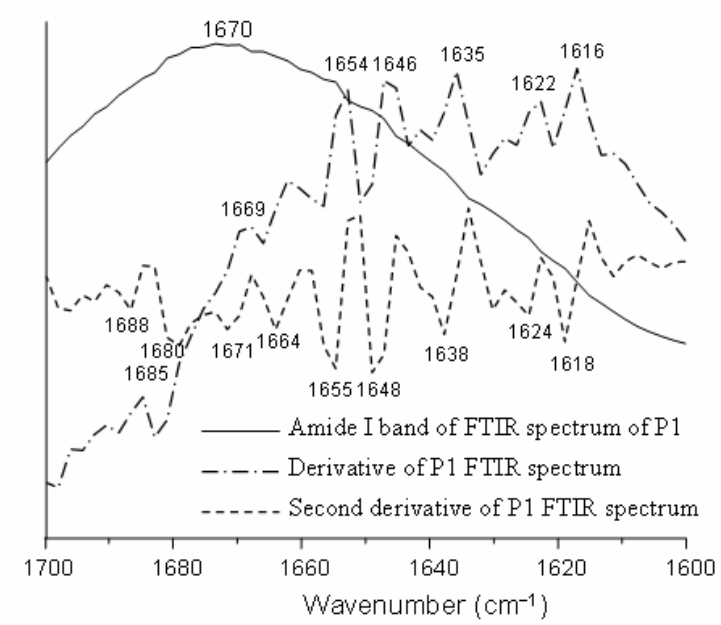

Fig. 3 – Amide I band in FT-IR spectrum of peptide GGGGHGGGGHGGGGHGGGG (P1) as well as its first and second derivatives.

Deconvolution of FT-IR spectra

We compared the results obtained by FT-IR with those of circular dichroism measurements and the predicted values for the proportion of conformers from K2D2 program, which is available online.[15] FT-IR spectra of peptide P1 showed a high proportion of unstructured populations, alongside with α-helix and β-type strands. The second derivative suggested a real but small proportion of β-sheet conformers. From Table 2 it is noted that the deconvolution of circular dicroism spectra using the program installed on Jasco-715 instrument leads indeed to appreciable values for unstructured conformers (unordered), which are found together with α- and β-populations. The table showed no β-sheet forms. The deconvolution of CD spectra using the K2D2 software resulted in 30% α-helical populations and 14% β forms, which is fully consistent with the values obtained by FT-IR. Since CD spectra were carried out in solution at pH 7.4, whereas the FT-

IR ones in the solid state, peptide conformation may weakly change in the transition process from one environment to another.

When used K2D2 software,[15] a predicted value of 9.55% α helix and a 44.29% β strands were found. These results are consistent with the signals from the IR spectrum of peptide P2, since this peptide has a major band with a maximum at 1632 cm^{-1} and another one at 1670 cm^{-1}, characteristic of β-type structures. From FT-IR spectrum the absorption at 1655 cm^{-1}, attributed to α-helical structures α-helix was hardly noticed. However, the second derivative contains a negative maximum at this wavenumber, corresponding to α-helical structures.

Aβ1-40 showed a large proportion of β-sheet conformers, as observed in FT-IR spectrum (1628 cm^{-1}). The same spectrum (Fig. 1) displayed some proportion of α-helix conformers (1656 cm^{-1}). Interestingly, no α-helical structures have been calculated using the program installed on the instrument. These helical populations were predicted only by the K2D2 software, as recommended by Iratxeta and Perez-Andrade-Navarro.[15] Thus, a proportion of 8.02% α helix and 22.14% β strands was calculated for Aβ peptide.

CD experiments

Albumin is a α-helical and β-turn protein containing 29.0% α-helix and 34.1% β-turn conformers (Table 2). No β-sheet conformers were calculated for ovalbumin. Instead, Aβ1-40 peptide proved to have a large content of β-sheet conformational isomers and a lower one of β-turn. Indeed, ovalbumin showed a characteristic spectrum for α-helical proteins with a maximum in the positive region of the spectrum at 185 nm and two negative maxima at 208 and 222 nm, respectively. On the contrary, Aβ1-40 displayed a large absorption band in the negative region at 198 nm. Aβ1–40 was found as a mixture of random coil (49.00%), β-sheet (33.7%), and β-turn (17.3%) forms (Table 2).

The peptides P1 and P2 had a low structural level due to their high content of non-chiral amino acid glycine (Fig. 4). Thus, both negative and positive contributions in CD spectra of the two peptides are very low. The large positive band in the region from 182.5 nm to 196.5 nm, with a maximum at 185 nm in the spectrum of P1 and a higher one from 191 nm to 205 nm in that of P2 suggest a high proportion of α-helix conformers in the aqueous solutions of both peptides. P1 had also several peaks in the negative region of the CD spectrum, such as those at 201.0, 209.5, and 218.5 nm, corresponding to the presence of α-helical conformers. However, the helical content of the two peptides proved to be different, as seen in Table 1 (P1 – 27.1% and P2 – 78.1%). An almost similar proportion of β-turn conformers seems to be correlated to the presence of histidine residues.

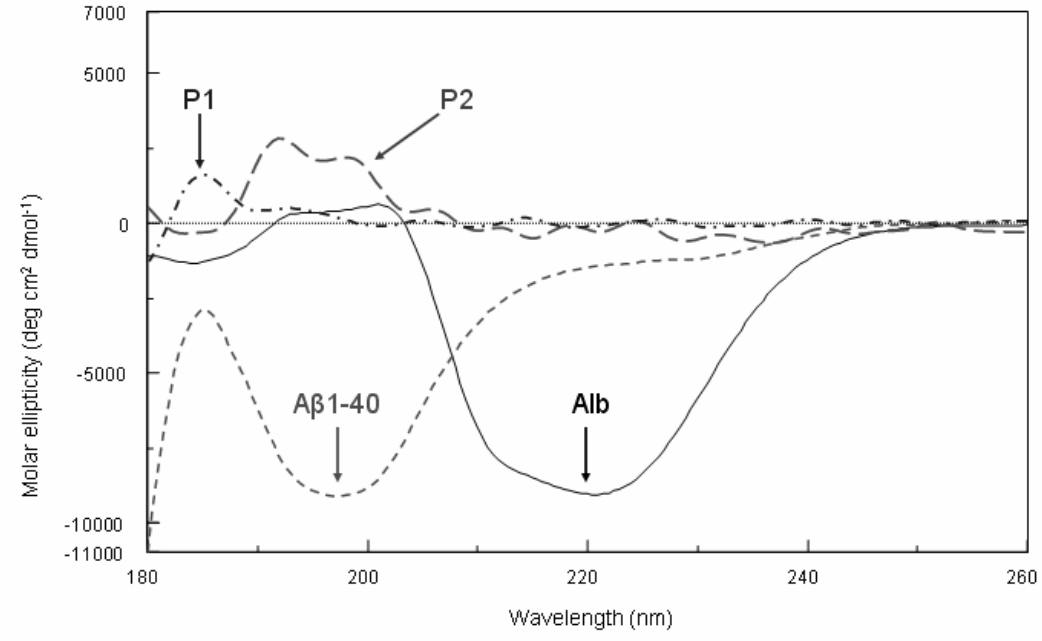

Fig. 4 – CD spectra of P1 and P2 peptides in water as well as those of amyloid-β peptide (Aβ1-40) and ovalbumin (Alb).

Table 2

Conformation of some peptides and ovalbumin (Alb) as revealed by CD spectroscopy

Conformation	P1	P2	Aβ1-40	Alb
Helix	27.1	78.1	0.0	29.0
Beta	0.0	0.0	33.7	0.0
Turn	21.5	21.9	17.3	34.1
Random	51.4	0.0	49.0	36.9

When used K2D2 software,[15] the proportion of each conformer was in a better agreement with the FT-IR bands.

Discussion

Our data show that the FT-IR spectra of the investigated peptides and protein have much in common, still important differences were observed especially in the amide I region.

Other spectroscopic techniques useful for studying protein structures in solutions are circular dichroism (CD), ultraviolet absorption and fluorescence spectroscopy, Raman and nuclear magnetic resonance (NMR).[16,17] Among the techniques for secondary structure, NMR and Raman spectroscopy need unusually high concentration of proteins, and NMR analysis is still limited to small proteins of about 200 amino acid residues. Circular dichroism (CD) is an excellent tool for rapid determination of the secondary structure and folding properties of proteins as well as to determine whether an expressed, purified protein is folded, or if a mutation affects its conformation or stability.[19] However, CD analysis is limited to clear protein solution (as opposed to membrane proteins) due to the problem with light scattering. Furthermore, for estimation of secondary structure of protein by CD accurate protein concentration is needed. Infrared spectroscopy is an emerging technique for protein analysis. Amide I, II and III are most commonly used IR spectral regions used for protein structure-function analysis. Recent advances in the development of instrumentation (Fourier transformation, sampling), protein IR data bank (band assignments to different components of secondary structure), and techniques (two-dimensional IR methods, time-resolution, and isotopic labeling) have significantly augmented IR spectroscopy as an analytical tool for peptides and proteins.

Two bands, 1655 and 1545 cm^{-1} can be assigned to α-helix structure, whereas those at 1630 and 1530 cm^{-1} can be associated with β-sheet conformation. Consequently, some authors found a positive correlation between the α-helical content of a protein and its absorption at 1545, 1655 and 1613 cm^{-1}, respectively.[18] They also showed a positive correlation between the β-sheet content and the corresponding absorbance at 1634 and 1691 cm^{-1}, whereas the β-sheet proportion was found negatively correlated with the absorbance at 1656 cm^{-1}. Therefore, the secondary structure can be predicted using some equations in which the absorption intensity of infrared light at various wavenumbers is correlated with the proportions of conformers.[18]

Characteristic vibrational bands in the amide I region of FT-IR spectra are different as a function of experimental conditions.[14] Thus, antiparallel β-sheet appeared at 1692 cm^{-1} in water, at 1690 cm^{-1} in trifluoroethanol, and at 1698 cm^{-1} in the solid state. Besides, in the solid state 1685 cm^{-1} and 1698 cm^{-1} are characteristic to β-turns, 1655 cm^{-1} to α-helix, 1636 cm^{-1} and 1623 cm^{-1}, is assigned to β-sheet, and 1611 cm^{-1} and 1604 cm^{-1} to aggregated strands.

The FT-IR spectroscopy is complementary to the CD-spectroscopy method according to the conformational studies of peptides and proteins.[14] Therefore, sometimes they may be used simultaneously. However, in the FT-IR spectroscopy in water solution to have a good signal-to-noise ratio, the concentrations of the peptide is usually from 4 to 30 mg/ml and it should be marked, in current studies for CD-spectroscopy the concentration of peptide is relatively low (0.2 mM).

The second derivative spectrum gives a negative peak for every band maximum or shoulder in the absorption spectrum whereas the fourth derivative spectrum gives a positive peak for every band maximum or shoulder.[20,21] However, the derivatization process does not preserve the integrated areas of individual components. Additionally, these techniques greatly amplify those features in the spectra originating from random noise or uncompensated sharp water vapor band components. Thus, the resolution enhancement should be performed on spectra with a high signal-to-noise ratio and after complete

elimination of water vapor bands. The use of resolution enhancement spectra for estimation of the secondary structure components in proteins is an established technique.[21,22] The resolved band components are assigned to α-helix, β-sheet and β-turn conformations.

Second derivative spectra allow the identification of various secondary structures present in the protein.[21] Most of the peak positions are easily found in the second derivative spectra. An improved method for carrying out second derivative analysis has established the utility of the method for obtaining quantitative as well as qualitative determination of α-helix, β-sheet, random and turn structures.[23] A curve fitting procedure can be applied to calculate quantitatively the area of each component representing a type of secondary structure. [8,22,23] Fig. 2 shows the second derivative spectrum of P1 and P2 peptides as well as those of amyloid-β peptide (Aβ1-40) and ovalbumin (Alb), which was obtained according to the methods of Dong *et al*.[23] The manipulation procedures were also carried out in accordance with Dong *et al.*

Biomedical applications

Our data show relevant changes in FT-IR spectra as a result of conformational changes in proteins and peptides. Even a small shift of histidine residues in the peptide backbone in the case of P1 and P2 resulted in significant changes of FT-IR spectra. In addition, environmentally-related conformational changes of biologically relevant proteins may clearly appear in the FT-IR spectra, especially in the second derivative ones. Therefore, we consider the effectiveness of this spectroscopic technique for biomedical applications.

It is also well-known that FT-IR spectra show differences between cancerous and non-cancerous parts of the analyzed tissues.[24] Additionally, the use of a two-band criterion showing an association of FT-IR-based spectral characteristics with clinically aggressive behaviour in prostate cancer manifest as local and/or distal spread was presented. Generally, the potential for the use of spectroscopic analysis for the evaluation of the biopotential of CaP in an accurate and reproducible manner has been demonstrated.[25,26] Quantitative analysis of the secondary structure in the amide I region shows different ratio of α-helix, β-sheet, β-turn and random coils, which is important and significant for spectral diagnosis of the level of malignancy.[27]

Infrared, Raman and UV-visible spectroscopic techniques were used to investigate the chemical mechanism of binding of the marker dye Congo red to deposits of Alzheimer's amyloid in cerebral tissue.[28] The combined tau/β-amyloid$_{1–42}$ quotient obtained by FT-IR was able to distinguish healthy from diseased AD subjects with 99% sensitivity and 86% specificity.[29]

CONCLUSIONS

The advantages and drawbacks of FT-IR spectroscopy as compared to CD one for structural characterization of peptides have been highlighted. Although, FT-IR spectra of small peptides can be highly complex and extremely difficult to interpret, they are concordant with the information obtained from CD spectroscopy. Amide bands associated with different secondary structural elements in peptides can show large deviations from one protein to another. This is partly due to the fact small peptides are often highly flexible and are solvated to a greater extent compared to globular proteins. Peptide aggregation can also result in the appearance of additional bands in the amide I region that can be erroneously attributed to peptide structure. The second derivative of amide I-III bands is essential to establish the peptide conformation. These are amongst a host of factors that needs to be taken into consideration when interpreting infrared spectra of peptides.

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