SATURATION EFFECTS IN FTIR SPECTROSCOPY: INTENSITY OF AMIDE I AND AMIDE II BANDS IN PROTEIN SPECTRA

Jože Grdadolnik

National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia.
Tel.: +386 01 47 60 200, Fax: +386 01 42 59 244, e-mail: joze.grdadolnik@ki.si

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Abstract

In the present article the dependence between the film thickness of the BSA protein and the intensity and structure of the Amide I and II bands are presented. Protein films thicker than 4 μm are shown to be unsuitable for structural investigation using transmission infrared spectroscopy since the signal for Amide I is saturated. Saturation of the Amide I band in the protein film spectrum causes complete disruption in the structure of the Amide I band. Furthermore, the structure of the nearby Amide II band is also perturbed. Hence, in order to avoid such band distortions the protein film thickness should be below 4 μm, which corresponds to the absorption value of the strongest band in the spectrum below 1.2 absorption units.

Introduction

Several techniques provide information on peptide and/or protein structures. X-ray crystallography and multidimensional NMR spectroscopy are undoubtedly the most powerful techniques available to determine the precise spatial distribution of protein atoms. However, Fourier Transform Infrared (FTIR) spectroscopy is also becoming accepted as an increasingly useful tool for the structural analysis of peptides and proteins. The relationship between amide bands and peptide structure has been established for some time. This relationship has been demonstrated by both theoretical[1-7] and experimental studies.[8-22] There are a number of advantages that make infrared spectroscopy an attractive tool for structural studies of proteins. Probably the most important advantage is that the spectra of proteins can be obtained in a wide range of environments. Measurements can be performed on solids such as powders or films,[23-25] in aqueous solutions,[16,18,20,21] in organic solvents,[26] and in detergent micelles,[27] as well as in phospholipid membranes.[22] Information on the secondary structure of proteins can be derived from analysis of the intensity of the Amide I band. Therefore, FTIR spectroscopy offers the possibility of identifying the most physiologically relevant conformation of a peptide or protein.

Most methods used to determine the secondary structure of peptides and proteins concentrate on an analysis of the Amide I band, located between 1700 cm^{-1} and 1600 cm^{-1}. The Amide I band is composed mainly (around 80%) of the C=O stretching vibration of the peptidic bond. The band can be analysed by comparison with a calibration set of spectra from proteins with known structures by using factor analysis[28] or by decomposing the Amide I band into its constituents via spectral decomposition,[23,24,29,30] derivative spectroscopy,[13] Fourier deconvolution,[11,20,21] or a band fitting algorithm.[8,9] All mentioned methods are based on examination of the structure of the Amide I band and the results of the analysis crucially depend on the shape of Amide I band.

The Amide I band is found next to the H_2O bands (OH stretching and OH bending), which are the most intense bands in the protein spectra when the protein is dissolved in water or buffer solution. Due to its high absorption, the Amide I band is the band most exposed to anomalies connected with the non-linear response of the infrared detector. The non-linear response of the detector has a dramatic influence on the bandshape and consequently on the determination of secondary structure. According to the Beer-Lambert law, the maximum intensity of the bands in the spectrum is directly related to the concentration and thickness of the sample. The quality of the spectrum depends mainly on the S/N ratio, which increases with increasing intensity of the observed bands. Hence, the tendency to prepare thicker and more concentrated samples. Even with the use of MCT detectors, which have a much larger linear region compared with DTGS detectors, the most intense bands may very easily be saturated and therefore severely perturbed.

The intensity of the solvent in the solution spectrum must be observed carefully in order to be able to perform a reliable subtraction. For this purpose an ATR (attenuated total reflection) cell with reduced pathlenght should be used when the maximum intensity of any peak exceeds 1.1 absorbance units. Only by using the reduced ATR cell (the degree of reduction depends on the solvent absorption) is it possible to carry out the proper subtraction. Proper subtraction allows us to use the whole mid infrared spectral region for detailed examination of the protein; this is also the only way to obtain complete information from the spectrum with no interference from solvent bands. However, the use of ATR techniques requires the calculation of the optical constants

(n, k) before subtraction in order to avoid artefacts in the absorbance spectrum due to reflection distortions.[31]

In the present article I will demonstrate how distortions in the Amide I band in the spectrum of Bovine Serum Albumin (BSA) are a consequence of band saturation. The BSA protein was measured in the form of films of varying thicknesses. The typical characteristics of such distortion effects and the procedure for avoiding such artefacts will also be shown.

Material and methods

BSA (MW= 66 kD) was used without purification. Films were prepared from the BSA solution in phosphate buffer (0.4 g/mL, pH = 7.5). The film density was determined using a picnometric method (ρ=1.51 g/cm^3). Self-standing films, used for the evaluation of a thickness calibration curve, were prepared on teflon-coated aluminium. For calibrating the film thickness, the spectral area between 3003 and 2887 cm^{-1} (with a linear baseline between 3003 and 2887 cm^{-1}) was used. These narrow bands are due to methyl and methylene vibrations of protein side-chains. Because of their moderate intensity and very weak overlapping with bands sensitive to hydration (NH stretching) these bands are very suitable for use as a calibration standard. The thickness of self-standing films was calculated from the interference fringe.[32] For this purpose an empty liquid cell with CaF_2 was used. Instead of the usual spacer, three pieces of protein film were put between the windows. To ensure homogeneity of film thickness, only those interference spectra with intense fringes were used for further thickness calculations. Each calculated thickness is an average of several independently measured experimental spectra with different parts of the films used as spacers. Compared to self-standing films, cast films are mechanically more stable and are not sensitive to hydration induced film twisting. The thickness of cast films was determined from the calibration curve.

BSA solutions used for ATR measurements were prepared by adding an appropriate amount of phosphate buffer (10 mM) to an already prepared solution (0.4 g/mL) in order to yield a 2.4 mM solution. Several parallels were made in order to avoid the possible presence of bubbles inside the cell, which can dramatically change the ATR spectrum. For the ATR measurement we used an adapted Specac CIRCLE ATR cell with a ZnSe rod crystal. The length of the crystal, which is exposed to measure liquid,

was shortened to prevent saturation effects, especially in the case of strong absorbing species such as water, various buffers, and dilute water solutions. Details on the alignment of the cell, as well as the determination of the number of reflections, are described elsewhere.[33] The optical constants (n and k) were calculated using the procedures proposed by Bertie and Eysel[34] and Bertie and Lan.[35] The spectra were recorded from 650 cm^{-1} to 8500 cm^{-1}. The part of spectrum between 0 cm^{-1} and 650 cm^{-1} was missing due to ZnSe absorption and was replaced with a linear descending function. Infrared spectra were recorded on Nicolet - Magna - 760 FTIR and Perkin-Elmer 2000 spectrometers equipped with a liquid nitrogen cooled MCT detector. 128 interferograms (films) and 1024 interferograms (buffer solutions) were collected and apodised with the Happ-Ganzel function. The spectral resolution is 2 cm^{-1}.

The overlapping bands were deconvolved and derivatized with GRAMS software. The Razor program package was used for band fitting. Maximum entropy was implemented to ensure slower relaxation and better convergence of the solutions. The sum of the Gaussian and Lorentzian functions was used throughout. Other functions were also examined but the results were inferior. Halfwidths, intensities, frequencies and part of both functions were allowed to vary in the iteration process. The initial values for band intensities and frequencies were derived from the deconvolution and second derivative spectroscopy.

Results and discussion

Three different spectra of BSA are presented in Figure 1. The first one (Figure 1a) belongs to the spectrum of BSA dissolved in phosphate buffer. The spectrum of the phosphate buffer is subtracted in order to reduce the overlapping between the OH bending and Amide I band. Since the ATR set up was used, the optical constant and absorption spectra were calculated for both spectra prior to subtraction as described in the materials and methods section. After subtraction of the phosphate buffer the Amide I band is the strongest in the spectrum. The absolute intensities of both bands are 0.163 a.u. and 0.096 a.u., respectively. Both bands are narrow with a halfwidth of 45 cm^{-1} (Amide I) and 42 cm^{-1} (Amide II), respectively. The presented spectrum is a typical spectrum for a protein in a buffer solution and will therefore serve as a model for the typical shape of the Amide I and Amide II bands. The chosen concentrations were used

to avoid too high intensity of the Amide I and Amide II bands, while a reduced ATR cell was used to avoid the saturation of the OH stretching band, thus allowing proper subtraction to be carried out.

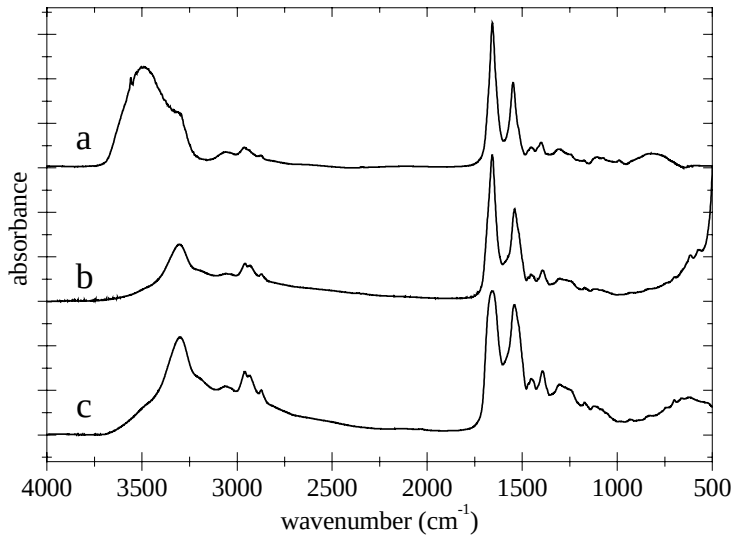

Figure 1. Infrared spectra of BSA protein. a) Calculated absorbance spectrum of 2.4 mM BSA solution in phosphate buffer (phosphate buffer is subtracted), b) Absorbance spectrum of 0.86 μm thick BSA cast film and c) Absorbance spectrum of 6.81 μm thick BSA cast film. Films are approximately scaled to the same value of Amide I absorption.

The second spectrum (Figure 1b) represents the infrared spectrum of a BSA cast film with a thickness of 0.86 μm. Only small spectral changes can be observed when the second spectrum is compared to the first one. These changes are mainly due to the slightly different state of hydration and there are, therefore, small changes in the hydrogen bond network within the protein and in its secondary structure.[25,36] The absolute intensities of Amide I and Amide II bands are 0.376 a.u. and 0.249 a.u., respectively, with corresponding halfwidths of 44 cm^{-1} and 50 cm^{-1}, respectively. The third spectrum (Figure 1c) belongs to the absorption spectrum of the cast film, which is much thicker than the previous one presented in the Figure 1b. The film thickness determined interferometrically and from the calibration curve is 6.81 μm. The spectrum features resemble the previous spectrum in almost all details except the shape of the Amide I band and the relative intensities of the Amide I and Amide II bands. Since the absorption due to a longer optical path is stronger, the corresponding intensities are 1.556 and 1.416 a.u. for the Amide I and Amide II band, respectively. However,

determining the differences between the spectra in Figure 1 can be made much easier by subtraction (Figure 2).

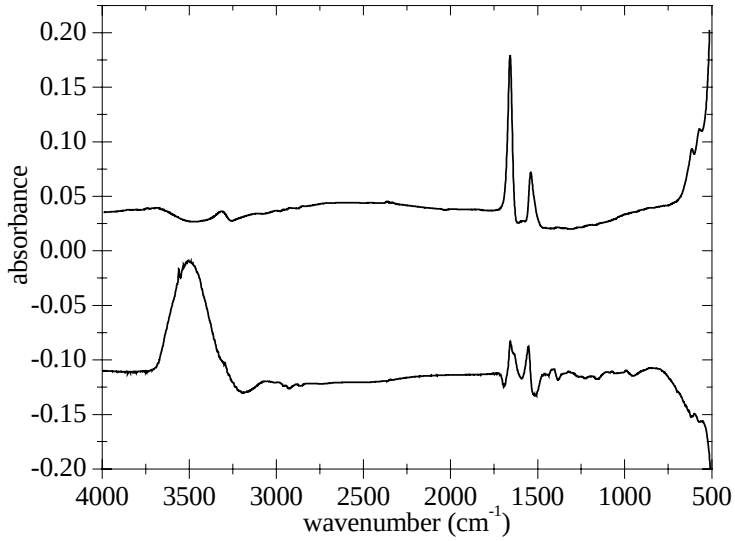

Figure 2. Top spectrum: a difference spectrum b-c from Figure 1. Bottom spectrum: a difference spectrum a-b from Figure 1.

The bottom difference spectrum in Figure 2, which belongs to the difference between the thin BSA film and the BSA dissolved in phosphate buffer, corresponds to typical spectra where the hydration number is changed.[23-25,32,37] The band in the high frequency region belongs to the absorption of the OH groups of hydrating H_2O molecules, which is much more intense in the case of the solution. Stronger hydration causes slight conformational changes in protein structure,[25] which can also be observed in the Amide A, Amide I, Amide II and corresponding Amide III band regions as dispersion-like curves. These features show that conformationally sensitive bands changed intensity, frequency and/or bandshape due to more extensive hydration. What is important is the fact that we can observe changes in all regions characteristic for protein as well as for H_2O vibration. All these changes can be easily interpreted as changes in the H-bond network in the protein as a consequence of the different state of hydration.

The totally opposite case is found in the upper spectrum where two different thick films (b and c spectrum from Figure 1) are subtracted. Since the hydration number of both spectra is very similar, we expect to observe a spectrum similar to the upper one although with less intense OH stretching bands and a corresponding response in the

bands of the protein groups. However, only two bands can be found in the whole middle infrared regions. These two bands are the remainders of Amide I and Amide II bands after subtraction. Their pure absorption character and solitariness in the spectrum are quite remarkable. It is well known that if we can find one vibration of a particular group in the difference spectrum (for example, the Amide I vibration of a peptide group) then we should be able to find all other vibrational bands connected to this particular group. In our case, one should also find the Amide A band (overtone, 3060 cm^{-1}), Amide II and Amide III (1300-1240 cm^{-1}) band as pure absorption bands or as dispersion like bands, similar to those seen in the bottom spectrum of Figure 2. If one does not, something is likely wrong with the linearity of the ordinate scale, i.e. with the linearity of the detector. In other words, some particular vibrations of the group can be eliminated and others not.

However, the relative intensity of the Amide I and Amide II bands is not the only thing that has changed in the thick film spectrum. The shape of the Amide I band is completely different. Therefore, the structure of the Amide I and Amide II bands were carefully examined using spectral resolution techniques. The complete structure of all three Amide I and Amide II bands is presented in Figures 3a, b, and c. Three independent methods were used for component determinations: Fourier deconvolution, derivative spectroscopy, and band fitting[36].

Frequencies for particular components, as well as the area as a percentage (%) of the whole Amide I region for all three type of different protein spectra, can be found in the Table 1. The presented band decomposition usually serves as a method for secondary structure determination. The proportion of the integrated intensities of particular components to the whole area of the Amide I band is then related to the structure of the protein. From Table 1 it is obvious that the BSA in solution (Figure 3a) and BSA in thin cast film (Figure 3b) have almost the same secondary structure. A similar outcome was expected for the thicker film. However, the decomposition of the Amide I bands (Figure 3c) shows us that the structure of the latter should be completely different. Besides the intensities, the position of the bands changed dramatically. We have to emphasise that the only difference between the two films is the thickness, and yet, according to the present study, only the thinner film has a structure comparable to that of the protein structure in solution. Perturbations in band structure can also be observed for the Amide II band (Figure 3c). In the BSA solution and /or thin film spectra only one component is

dominant, while in the spectrum of the thicker film there are two components with practically the same intensity.

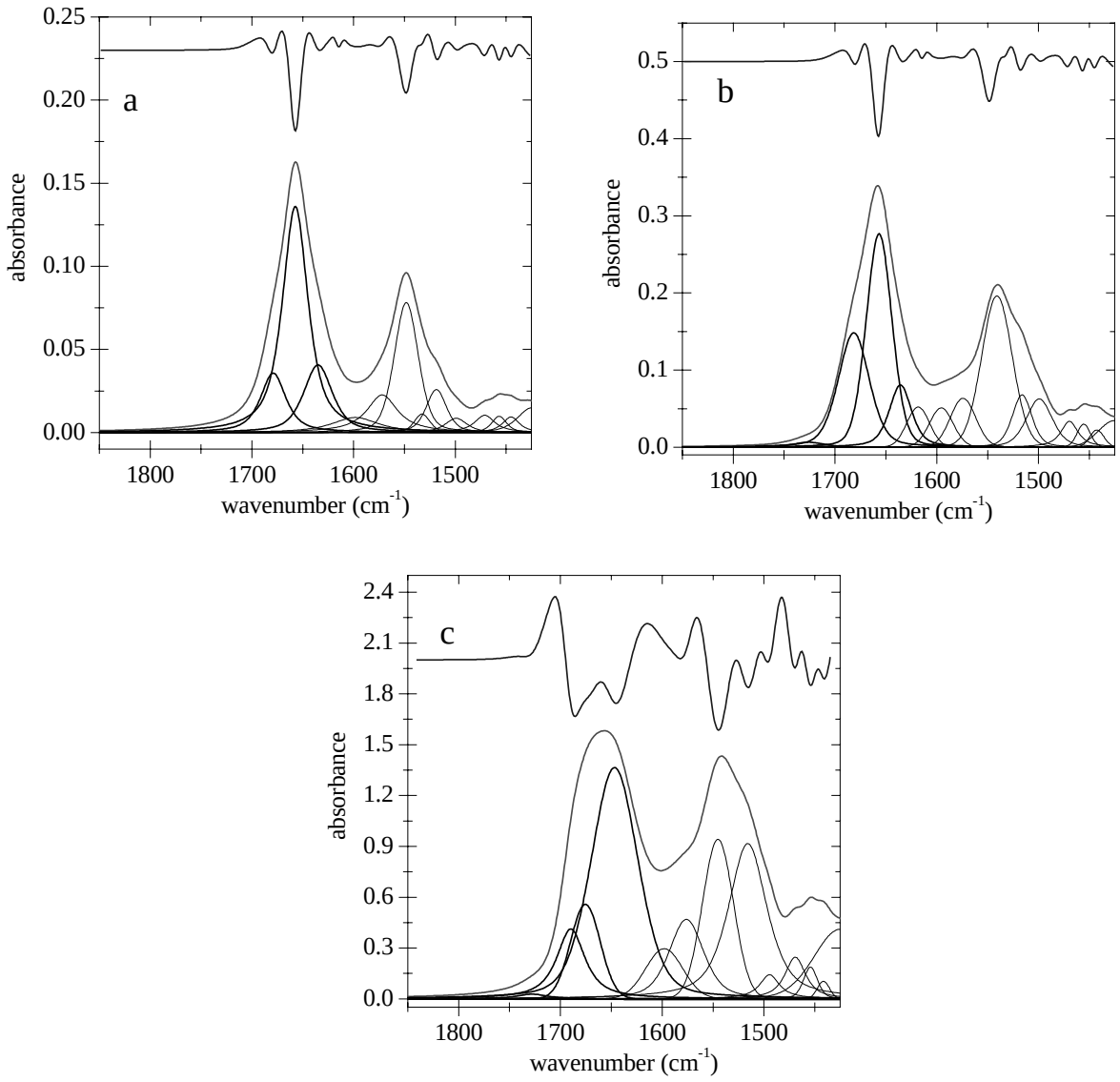

Figure 3. Band fitting of Amide I and Amide II region in: a) 2.4 mM BSA in buffers solution, b) 0.89 μm thick BSA cast film, c) 6.81 μm thick BSA cast film. Upper spectrum in all figures is the second derivative spectra of the same regions.

In Figure 4 the ratio of the Amide I and Amide II band intensities as a function of film thickness is shown. The ratio is around 1.5 until the thickness becomes larger than 4μm. For thicker films the Amide I / Amide II ratio drops to almost 1. Furthermore, the change in the Amide I band shape has the same functional dependence as the Amide I /

Amide II intensity ratio. For film thicker than 4 μm the shape is the same as that presented in Figure 1b and corresponds to the spectrum of BSA dissolved in phosphate buffer (Figure 1a), which represents the native conformation of BSA.

Table 1. The frequencies (cm^{-1}) and percentage (%) area of the whole Amide I band of components are presented in Figure 3.

	Component 1		Component 2		Component 3		Component 4	
	Frequency	Area (%)	Frequency	Area (%)	Frequency	Area (%)	Frequency	Area (%)
BSA solution	1679	21.8	1657	57.1	1635	22.1	1614	0
BSA film 0.86μm	1681	25.8	1657	55.2	1636	12.4	1618	7.6
BSA film 6.82μm	1690 1675	15.4 16.1	-	-	1647	68.5	-	-

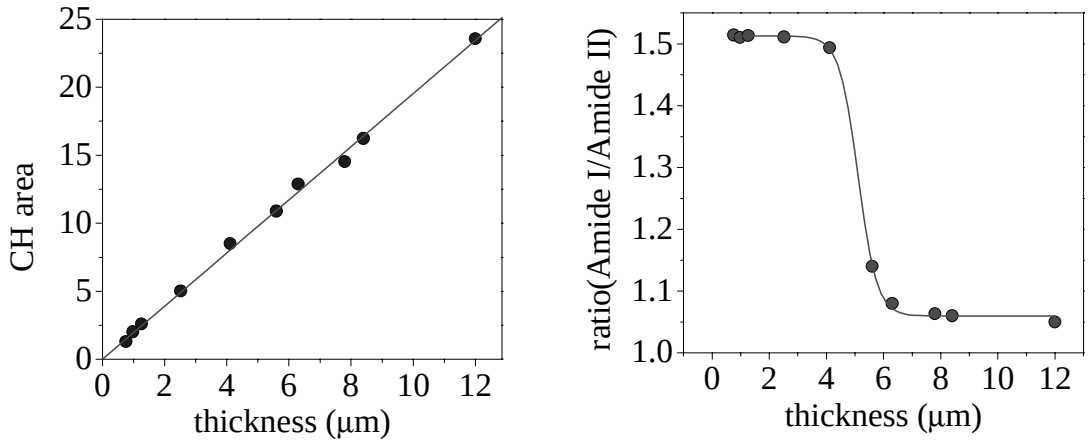

Figure 4. Left graph: The intensity ratio of Amide I / Amide II band. Right graph: Calibration curve for thickness determination of cast BSA films.

The difference spectra presented in Figure 2, as well as the study of the band structure presented in Figures 3, implies that the reason for all of the disturbances in the thicker films is saturation of the absorption due to detector overload. At high intensities in the absorption spectrum (higher that 1.2 a. u.) the detector is functioning in its nonlinear region meaning that the most intense band is cut (in our case this is the Amide

I band), consequently reducing the relative Amide I/Amide II intensity to values around 1.

Another piece of evidence that makes a determination of the band shape distortion due to the saturation effect even more pronounced is the calibration curve for protein thickness. Although the Amide I band is saturated, the CH stretching area near 3000 cm^{-1} can still be used to determine film thickness. This means that, firstly, other parts of protein spectrum within the Amide I and Amide II region are not influenced by the effect of the nonlinear response of the detector. Secondly, almost perfect matching of a point (R=0.99751) with the straight line (y=1.954*x) implies that the measured intensities in the CH region are well inside the Beer-Lambert law and hence an ideal tool for thickness determination. We must bear in mind that only the strongest bands are influenced by severe detector discontinuity.

Conclusions

In the present article the structure of the Amide I and Amide II bands in a BSA solution in buffer and in films of the same protein cast on the windows were compared. Matching of band structure is excellent in the case of thin films and BSA dissolved in buffer. Small changes can be interpreted as a consequence of different states of hydration. The difference spectrum shows changes in the hydrogen bond network mainly between the H_2O molecules and particular parts of the protein (peptide bond units and side-chains). On the other hand, the structure of the amide bands in films thicker than 4 μm is quite different. On the basis of the presented band structure, one would be led to conclude that in thicker films the secondary structure is completely different. However, such dissimilar structure in the Amide bands is not caused by a radical change in the protein's secondary structure but is in reality an artifact created by the non-linear response of the detector. So-called saturation effects take place and disturb the band shape of the Amide bands. In cast or self-standing films the ratio between the Amide I and Amide II band should be higher than 1.1 and this can be achieved only for protein films thinner that 4 μm. Only when this condition is fulfilled can the Amide I band be safely used for structural investigation.

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Povzetek

Študirali smo vpliv debeline proteinskega filma na obliko in relativno intenziteto amidnega I traku v infrardečem spektru BSA proteina. Pokazali smo, da filmi, ki so debelejši kot 4μm, niso primerni za določevanje sekundarne strukture. Nasičenje infrardečega detektorja, ki je posledica predebelih filmov, popolnoma spremeni obliko in relativno intenziteto amidnega I traku.