分离科学与工程实验室论文集

SYMPOSIUM OF LSSE

（1994-2003）

编辑：魏晓芳

Editor:Wei Xiaofang

中国科学院过程工程研究所

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LABORATORY OF SEPARATION SCIENCE AND ENGINEERING

INSTITUTE OF PROCESS ENGINEERING

CHINESE ACADMEMY OF SCIENCES

二〇〇三年三月

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目 录

一. 1994 年发表的论文

二. 1995 年发表的论文

三．1996 年发表的论文

四．1997 年发表的论文

五. 1998 年发表的论文

六. 1999 年发表的论文

七．2000 年发表的论文

八. 2001 年发表的论文

九. 2002 年度发表的论文

十. 2003 年发表的论文（统计到 2003 年 3 月 12 日）

Extraction of lysozyme, α-chymotrypsin, and pepsin into reverse micelles formed using an anionic surfactant, isooctane, and water

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The extraction of lysozyme, α-chymotrypsin, and pepsin from buffered salt solutions into reverse micelles was examined at different pH values and surfactant concentrations. The reverse micelles were formed by mixing aqueous buffer supplemented with KCl and an organic phase of isooctane(2,2,4-trimethylpentane), containing the anionic surfactant, Aerosol O. T. (dioctyl ester of sodium sulfosuccinic acid). The technique of dynamic laser scattering was used to measure the size of reverse micelles which were in equilibrium with the aqueous phase. It was found that the size of the reverse micelles decreased with increasing ionic strength but increased with increasing AOT concentration. In the process of extraction, the reverse micelles might have rearranged themselves to host the protein. The sizes of protein-filled and -unfilled reverse micelles were different, and an open equilibrium could be reached between them. Under the extraction conditions, only a small number of micelles were found to contain protein.

Keywords: Reverse micelles; dynamic laser scatter; extraction; lysozyme; α-chymotrypsin; pepsin

Introduction

With the rapid development of biochemical engineering, highly selective and economical separation methods are becoming more and more important in commercial processes. Reverse micelles, which are the self-assemblage of surfactant molecules in organic solvent, provide a novel means for biomaterial separation.[1-3]

Reverse micelles have polar cores that can solubilize water. Enzymes and other biomaterial can also be hosted in the reverse micellar solution after the solubilization of water.[4] A simple structural model has been put forward that assumes that the enzymes are in the water core of reverse micelles.[5,6] The enzymes are surrounded by layers of water that prevent the enzymes from contacting the organic solvents that could cause the loss of activity.[7]

Although much work has been reported on the solubilization of protein in reverse micellar solution,[8] little is known about how the protein transfers from the aqueous phase to the organic phase in the process of reverse micelle extraction.

In this paper we report the extraction of lysozyme, α-chymotrypsin, and pepsin with AOT isooctane reverse micelle forming solution. By measuring the size of the reverse micelles that have or have not hosted protein after liquid-liquid extraction, respectively, we try to explain how the protein is hosted in reverse micelles.

Materials and methods

Materials

AOT (Aerosol O.T.: American Cyanamid registered trade name) was purchased from FLUKA and used without further purification. Isooctane was purchased from the market and was of CP grade. Lysozyme (MW = 14,300, pI = 10.9), α-chymotrypsin (MW = 25,500, pI = 8.5), and pepsin (MW = 34,000, pI < 1.0) were purchased from the Dongfong Biochemical Plant, Shanghai Institute of Biochemistry, the Chinese Academy of Sciences, BR. All inorganic reagents were purchased from the local market and were of analytical grade.

Preparation of samples

The organic phase was prepared by dissolving AOT in isooctane. The aqueous buffer solution consisted of citric acid–dibasic potassium phosphate for pH 2–8 and borate–sodium hydroxide for pH 9–12, with 0.02 M ionic concentration. All buffers used in the phase-transfer experiments contained 0.2 M KCl to promote the

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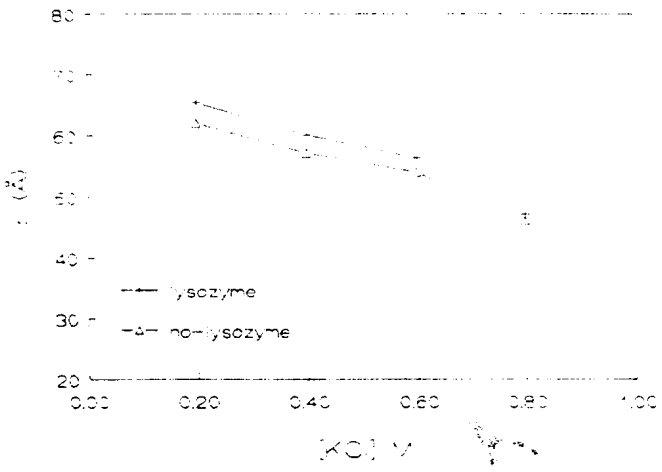

Figure 1 Average hydrodynamic radius of reverse micelles at different ionic strengths. [AOT/isooctane] = 100 mM; [lysozyme] = 1 mg ml^{-1}; pH = 5.4

formation of a two-phase system. The protein solution was prepared by dissolving protein in the buffer solution. The concentration in the aqueous phase was approximately 1 mg ml^{-1} before transfer. The phase transfer experiments were carried out in tightly stoppered 50-ml glass flasks. Two equivalent volumes (generally 5 ml) of each phase were contacted in a flask and agitated horizontally on an orbital stirrer at 250 rev min^{-1} for 7 min. Then the mixture was centrifuged for 15 min at 3500 rev min^{-1} and separated into two phases. The upper organic phase and the lower aqueous phase were separated and analyzed separately.

Analytical methods

The protein concentration was determined by UV absorption at 280 nm on a 751G UV/Vis spectrophotometer. The water content in the reverse-micelles-containing organic phase was determined by the Karl-Fisher method. W_0 is defined as the water content in the organic phase, which is the ratio of the molarity of water to the molarity of surfactant. $W_0 = [H_2O]/[\text{surfactant}]$.

The dynamic laser scatter experiment was carried out with a laser dynamic light scattering spectrometer set up by the Laboratory of Light Scattering, Peking University. The apparatus included a Spectra physics 124B Argon laser which was operated at 514.5 nm. Laser power ranged from 50 to 1000 mW. Reverse micellar solutions were prepared by liquid–liquid extractions, which were in equilibrium with the aqueous phase. Prior to analysis, the samples were passed through a Millipore 0.3-μm filter to remove any dust particles. Scattering data were collected at 25°C at a scattering angle of 90°.

The autocorrelation functions were inverted to obtain diffusion coefficients using a simple first-order cumulant method. The correlator assumed the Stokes-Einstein relation to obtain a particle diameter from a diffusion coefficient.[9]

Results and discussion

Structural characteristics of reverse micelles

The effect of the size of the reverse micelles on the mechanism of protein solubilization has been previously examined.[10-12] especially for the AOT/isooctane reverse micellar system. But the structural characteristics of reverse micelles are still unclear, especially for the reverse micellar system in liquid–liquid extraction processes.

The shell-and-core model of Bonner *et al.*[5] is usually used to interpret the solubilization of proteins in reverse micelles. It is proposed that the protein is surrounded by layers of water and the hydrophilic head groups of the surfactant are outside the water layers. The shape of this kind of reverse micelle may be considered as a sphere which has several shells. From this view, dynamic laser scattering can be used to determine the size of reverse micelles.

Figure 1 shows the effect of ionic strength on the sizes of reverse micelles in organic phase in equilibrium with the aqueous phase. The aqueous phase may contain lysozyme or contain no protein. Both curves decrease as the concentration of KCl rises. The most important effect of ionic strength is that it reduces the electrostatic repulsion between the surfactant head groups, resulting in a decrease in the size of the reverse micelles at higher ionic strength. This result can be demonstrated by *Figure 1*; both curves decrease as the concentration of KCl rises.

The concentration of surfactant also affects the size of reverse micelles.[12] With increasing AOT concentration, the size of reverse micelles increases, as shown in *Figure 2*. This may just illustrate one of the characteristics of reverse micelles, i.e., the size of reverse micelles varies with the concentration of surfactant. Micelle size in the aqueous phase, however, remains constant once the concentration of the surfactant exceeds the critical micelle concentration.

By analyzing the dynamic laser scatter data, it is also found that the dispersion of the reverse micelles increases with decreased AOT concentration. From these experimental data, we can point out that, with the concentration of all reagents, the protein-filled and -unfilled micelles reach an open equilibrium that varies easily. If the difference between the sizes of protein-containing and non-protein-containing reverse micelles is large, as shown by *Figure 2* in low AOT concentration, the system will become unstable and the equilibrium will be changed. Thus, one can observe that the dispersion increases with decreased AOT concentration.

According to the shell-and-core model, different values of W_0 always reflect different size of reverse micelles. If W_0

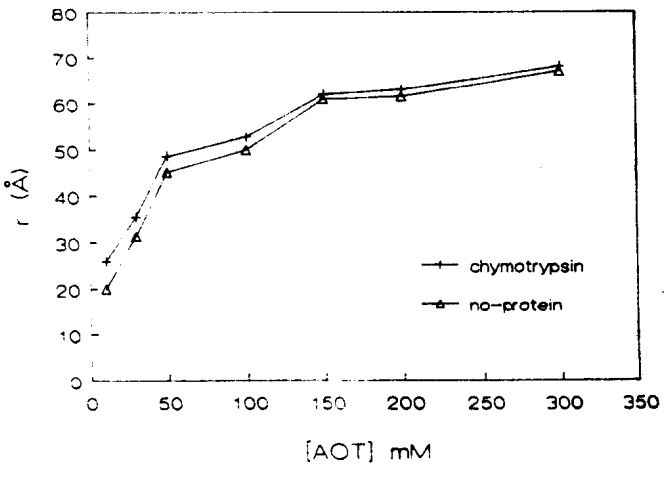

Figure 2 Average hydrodynamic radius of reverse micelles at different AOT concentrations [KCl] = 0.2 M; [α-chymotrypsin] = 1 mg ml^{-1}; pH = 5.4

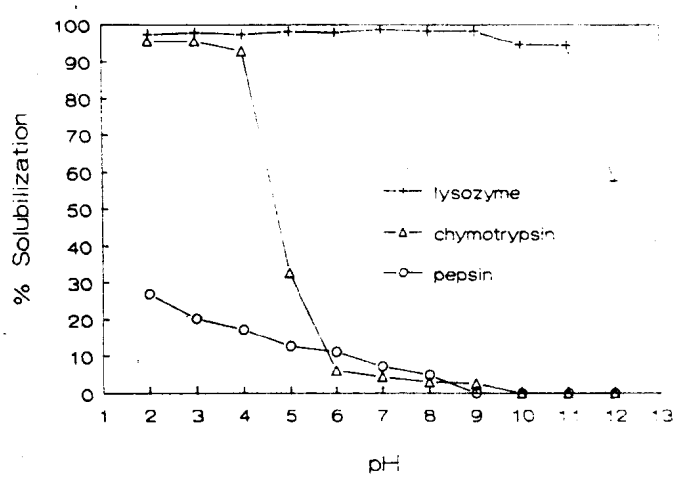

Figure 3 Effect of pH on the solubilization of three kinds of protein. [AOT/isooctane] = 100 mM; [KCl] = 0.2 M; [protein] = 1 mg ml^{-1}

in reverse micelles were measured at the same time as the sizes of reverse micelles, it would be reasonable to assume that there existed a relation between W_o and the average aggregate number (N_{ag}) of reverse micelles. In order to determine this kind of relation, Matzke's equation

$$N_{ag} = 32.1 - 1.25\,W_o + 0.873W_o^2$$

is quoted here. Collecting the data of W_o and N_{ag} from references 9, 13, 14, and then using this equation to calculate the values of N_{ag} from the experimental values of W_o, it is found that the error between the calculated values of N_{ag} and the experimental values of N_{ag} is small at low W_o (<10) but becomes remarkable at high W_o. In this paper, the above equation was used to calculate the aggregate number at low W_o.

Protein solubilization using liquid–liquid extraction

The factors that affect the extent of enzyme transfer to the reverse micellar solution can be divided into two groups: one is the electrostatic characteristics of the aqueous solution, e.g., pH, ionic strength, and type of salt present; the other is the parameters of organic phase, e.g., types of solvent and surfactant and concentration of surfactant. Among them, pH is believed to be one of the most important factors.

pH effects

For the AOT/isooctane reverse micellar system, significant transfer of protein will happen when the pH values are below the isoelectric point (pI) of protein. *Figure 3* shows the results of extraction of lysozyme, α-chymotrypsin, and pepsin. The properties of these proteins are listed in Materials. Pepsin, perhaps due to its low pI (<1) or its high molecular weight, only has a low extent of protein transfer within the experiment range.

Generally, pH is thought to be the dominant factor in the process of protein transfer by modifying the charge distribution over the protein surface.[15] But a recent study[16] has pointed out that the charge distribution over the protein surface is asymmetrical. This kind of asymmetry may change the electrostatic attraction between the protein and surfactant so that transfer can occur at pH values above the pI value of the protein for AOT reverse micelles system.

When the pH values decreased, the amount of white precipitate formed between the two phases increased. After analysis, it is found that it mainly contains protein and surfactant. These precipitates may lead to the loss of protein in the process of extraction, and make the extent of protein transfer decrease at low pH values. Some other groups have also found this phenomenon.[17]

Occupancy of protein in reverse micelles at different pH values

If the reverse-micelles-containing organic phase is in equilibrium with the aqueous phase, the water content of the organic phase is little affected by the pH values, as *Figure 4* shows. Then, the average aggregate number of reverse micelles can be calculated by using Matzke's equation, which has been mentioned above and is also shown in *Figure 4*.

Calculating the number of protein molecules in the organic phase by taking the concentration of protein in the organic phase (C_{org}) and dividing it by the number of reverse micelles present in the system allows the occupancy of proteins in the reverse micellar solution to be obtained, as shown in *Figure 5*. We find that in the experimental range, only a small number of reverse micelles contain proteins.

Conclusions

Reverse micelles were formed in a two-phase system to produce water-containing micelles that ranged in size from 20 to 70Å. The effects of increasing the ionic strength of the aqueous phase, using KCl (0 to 1.0 M), and altering the pH between 1 and 13 on protein transfer into these micelles were examined. Lysozyme transfer to reverse micelles increased gradually from pH 2 to 11, before decreasing sharply at pH 12. The transfer of α-chymotrypsin to micelles occurred from aqueous solutions with pH values between 2

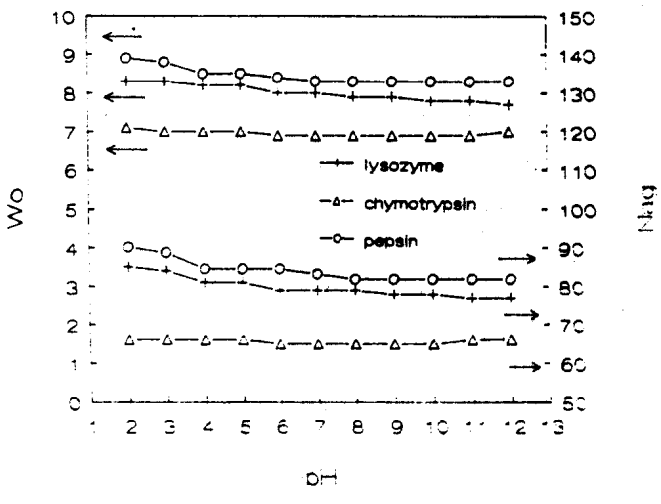

Figure 4 Effect of pH on the water content in organic phase and average aggregate number of reverse micelles. [AOT/isooctane] = 100 mM; [KCl] = 0.2 M; [protein] = 1 mg ml^{-1}

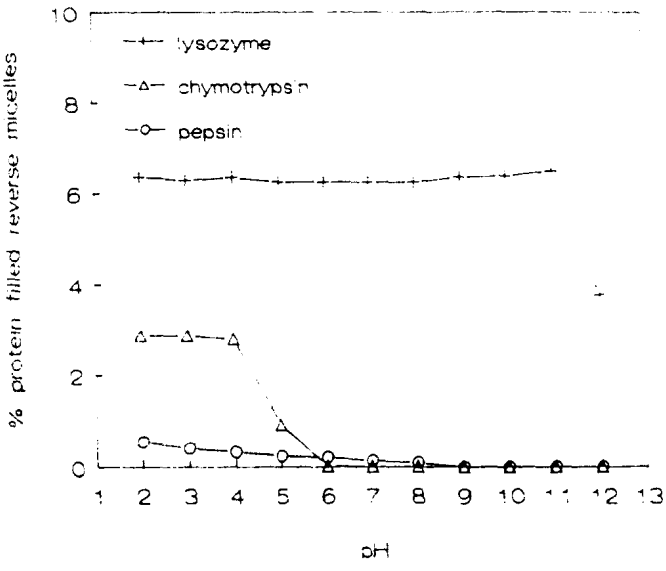

Figure 5 Occupancy of three kinds of protein in reverse micelles. [AOT:isooctane] = 100 mM; [KCl] = 0.2 M; [protein] = 1 mg ml^{-1}

and 4. Negligible amounts of pepsin were transferred at any pH. The quantity of lysozyme and α-chymotrypsin that could be transferred to these reverse micelles was low. Much of the protein formed an emulsion with the surfactant at the water isooctane interface. More than 90% of the micelles that were formed contained only water.

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Fourier transform infrared spectra studies of protein in reverse micelles: effect of AOT/isooctane on the secondary structure of α-chymotrypsin

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Abstract

The amide I region Fourier transform infrared (FTIR) spectra of α-chymotrypsin have been studied in deuterium oxide (D_2O) solution and also in reverse micellar solution of AOT/isooctane. The Fourier second derivative was applied to all spectra, revealing that the amide I band of α-chymotrypsin in D_2O and in reverse micellar solution consists of nine components. The band frequencies are assigned to α-helix, β-sheet, random and turn structure. The second derivative spectra of α-chymotrypsin have been shifted in the reverse micellar solution of AOT/isooctane in comparison with its spectra in D_2O. This shift has also changed the intensity of each band. Through accurate measurement of the band intensities, the relative amount of different structure of α-chymotrypsin can be estimated. The comparison of the calculated results obtained in D_2O with those obtained in reverse micellar solution provides the possibility to analyze the effect of reverse micellar solution of AOT/isooctane on the secondary structure of α-chymotrypsin. The results indicate that the reverse micellar solution has decreased the amount of α-helix and β-sheet structure and increased the amount of random and turn structure in α-chymotrypsin. The increase of the amount of random structure might loosen the structure of α-chymotrypsin and change the activity of the enzyme.

Key words: FTIR; Reverse micelle; α-Chymotrypsin; Secondary structure

1. Introduction

The reverse micelles are self-assemblage of amphiphilic molecules in apolar media [1]. The most conventional reverse micellar system studied is with AOT as amphiphic surfactant and with isooctane as organic solvent. These reverse micelles always consist of a polar core which includes the headgroup of surfactant surrounded by the hydrophobic surfactant tail protruding into the bulk organic medium. Under appropriate conditions, some polar materials, e.g., water, salts and in particular proteins (include enzymes), can be taken up by the micelles and thereby solubilized in an inhospitable organic medium [2]. Because enzymes can be hosted in reverse micelles without loss of activity, biotechnologists have shown great interest in recent years [3] with reverse micelles as versatile microreactors in which guest molecules can be brought to reaction with novel chemical properties. It can also be used to recover protein from the aqueous phase on a large scale through phase–phase extraction.

A considerable volume of work have been published on the protein dissolved in AOT/isooctane reverse micelles [4], but not much work has been reported about the effect of AOT/isooctane on the structure of protein [5], especially by using FTIR spectroscopy. The infrared spectrometry is a particularly suitable method to study the protein conformation and protein–ligand interaction as it does not require to insert any probe into the system and can be used for aqueous phase. The most important part of spectra for the infrared assessment of the secondary structure of protein is the region between 1600–1700 cm^{-1}, which contains the so called amide I bands [6]. The amide I bands, due almost entirely to the C = O stretch vibration of the peptide linkage to constitute the backbone structure are the most useful probe for determining the secondary structure of protein in solution [7]. However, the low sensitivity of conventional dispersive infrared instrument has severely limited the early experimental work. Now, with the high sensitivity of the FTIR instrument availablely makes it feasible to measure amide I region spectra for protein in

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solution. Furthermore, the development of methods for analysis of spectral data, e.g. Fourier self-deconvolution, second derivation and band curve-fitting [8], makes it possible to distinguish the individual components within the intrinsically overlapped amide I region band contours.Due to the problem of solubility, most of the studies on conformation of proteins in nonaqueous solvents either have been theoretical or have used a mixture of solvent and water [9]. In this paper, we have used the injection method to dissolve protein in reverse micellar solution. In this manner, we can study the distribution of secondary structure determined from amide I region spectra of α-chymotrypsin in the aqueous solution and reverse micellar solution respectively. The second derivative band areas (integrated intensities) give relative amounts of different types of secondary structure for α-chymotrypsin [7]. We have compared the calculated results in the reverse micellar solution to those obtained in the aqueous solution.

2. Materials and methods

2.1. Materials

Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) was purchased from Fluka (> 98%). Isooctane (AR) and D_2O (> 99%) were supplied by Beijing chemicals plant. α-chymotrypsin (BR) was purchased from the Shanghai Institute of Biochemistry, Chinese Academy of Sciences, and used without further purification.

2.2. Preparation of samples

Protein solution (5%, w/v) was prepared by dissolving α-chymotrypsin in D_2O. AOT was dissolved in isooctane with 100 mM concentration. Protein solution with concentration of 15% (w/v) was also prepared, and used for injection into the reverse micellar solution. A clear reverse micellar solution containing protein can be obtained simply by handshaking. The concentration of protein in the reverse micellar solution was about 0.1–0.5% (w/v); the water content Wo($[H_2O]/[AOT]$ mole ratio) in the solution studied was about 6–10. All solution of protein in D_2O were prepared 48 h before using in order to make sure the H–D exchange was completed [10].

2.3. Measurement of infrared spectra and data manipulation

The protein solution was placed in a cell with BaF^2 window and with optical path length of 15 μm. The reverse micellar solution was also placed in a cell with BaF^2 window but with optical path length of 100 μm. Infrared spectra were measured with a Nicolet 7199B Fourier Transform Infrared spectrophotometer at 20°C. For each spectrum 250 interferograms were collected with a 2 cm^{-1} resolution. Reference spectra were recorded under identical conditions with media containing no protein. The spectra of protein in reverse micellar solution were obtained by subtracting its spectra from those of the blank reverse micellar solution containing no protein. The signal-to-noise ratio of these spectra (≫ 500) was high enough to perform the manipulation of subtraction [10]. A half-bandwidth of 13 cm^{-1} and a resolution enhancement parameter (K) value of 2.4 were used to obtain the spectral deconvolution. Second derivation spectra were obtained with the same instrument. The relative amounts of different secondary structure of protein were determined from the infrared second derivative amide I spectra by manually computing the areas under the bands assigned to a particular substructure.

3. Results and discussion

3.1. General assignment of deconvoluted amide I components

The original amide I region spectra and the deconvoluted spectra of α-chymotrypsin in D_2O are shown in Fig. 1. The second derivative spectra derived from the original spectra are shown in Fig. 2. Nine bands can be observed in the deconvoluted spectra and also in the second derivative spectra. The frequencies of these nine bands in the deconvoluted spectra, under the parameters of $K = 2.4$ and a half-bandwidth of 13 cm^{-1}, are found to be essentially the

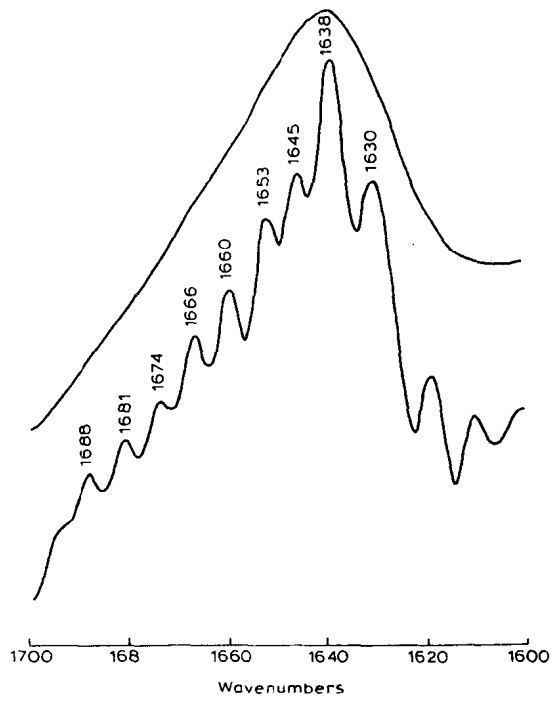

Fig. 1. Original spectrum and deconvoluted spectrum of α-chymotryp in 5% D_2O. Deconvolution parameters HW = 13 cm^{-1}, K = 2.4.

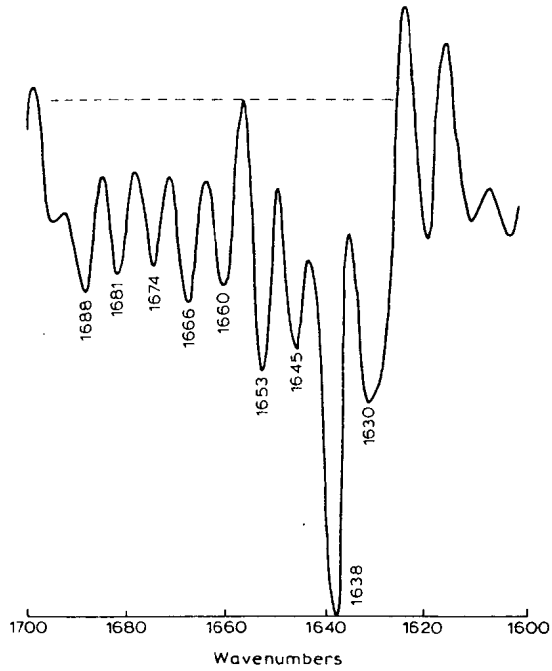

Fig. 2. Second-derivative spectrum of α-chymotrypsin in 5% D_2O.

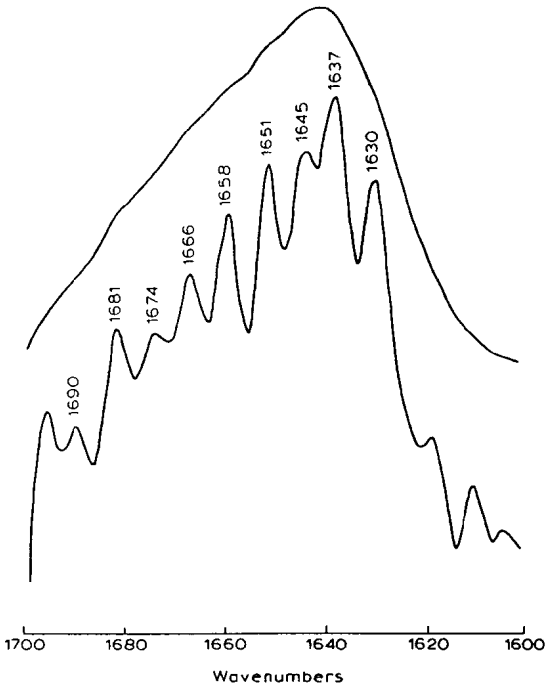

Fig. 3. Original FTIR spectrum and deconvoluted spectrum of α-chymotrypsin in AOT/isooctane reverse micellar solution. Decovolution parameters HW = 13 cm^{-1}, K = 2.4.

same as in the second derivative spectra, as shown in Table 1.

After subtraction, the amide I region spectra of α-chymotrypsin in reverse micellar solution can be obtained, as shown in Figs. 3 and 4. Nine bands can be separated from both the deconvoluted spectra and the second derivative spectra. Detailed information can be obtained by analysing the deconvoluted spectra or second derivative spectra. In this paper, all band assignments and calculations are all based on the second derivative infrared spectra.

The frequencies of these nine bands are shifted in the reverse micellar solution by comparing with frequencies obtained in D_2O. Results indicate some shifted to higher wave number, some shifted to lower while some have shown no change as listed in Table 2. The shift of bands in amide I can be originated only from the change of α-chymotrypsin structure in the reverse micellar solution. At least three factors contribute to the changes in amide environment of α-chymotrypsin. First, the unusual properties of water localized in the interior of reverse micelles [17] could bring about stronger interaction with the charged groups of amino acid on surface of the neighboring α-chymotrypsin molecule than in the aqueous phase. The strong interaction of D_2O with the molecules of α-chymotrypsin increases the effect of hydrogen-bonding, and causes a decrease in band frequency of amide I (hydrogen-bonding in model systems always leads to a decrease in frequency [18]). Second, the interaction of AOT and α-chymotrypsin can change the structure of α-chymotrypsin, and also the band frequency in amide I spectra. Third, the contact of α-chymotrypsin molecules with isooctane in the process of solubilization can also change the structure of α-chymotrypsin. Besides, solvent (e.g., isooctane) of low dielectric constant can also shift the band to a lower frequency too [19]. The co-effect of these three factors has changed the amide environment, and makes most of the bands shift to lower frequencies or stay unchanged, only one band has been shifted to higher frequency in reverse micelles.

3.2. Assignment for α-helical structure

There is only one band in amide I region for α-helix with frequency around 1650–1660 cm^{-1} [11,14]. By analysing the nine bands as indicated in Fig. 2, 1653 cm^{-1} band is assigned to α-helical structure. Different proteins with α-helical structure have shown different amide I

Table 1
Amide I frequencies for α-chymotrypsin in D_2O (cm^{-1})

	α-helix	β-sheet	Random coil	β-Turn
Deconvoluted spectrum	1653	1674, 1638, 1630	1645	1688, 1681, 1666, 1660
Second derivative spectrum	1653	1674, 1638, 1630	1645	1688, 1681, 1666, 1660