Effect of Reverse Micelles on the Secondary Structure of α-chymotrypsin and Subtilisin Carlsberg by FTIR Spectroscopy

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Abstract

In this paper, we took α-chymotrypsin and Subtilisin Carlsberg as model proteins to investigate the effect of reverse micelles (AOT /isooctane and CTAB/ n-hexanol /hexane) on the secondary structure of proteins by FTIR spectroscopy. α-chymotrypsin showed a decrease in α-helix content from 9% to 6.2% and a decrease in β-sheet content in AOT/isooctane reverse micelles which implied a more loose and irregular structure. But α-chymotrypsin showed an increase in α-helix content and a mini decrease in β-sheet content which implied a more regular structure. Subtilisin Carlsberg showed an increase in α-helix content and an increase in β-sheet content in AOT/isooctane reverse micelles, which implied a more regular structure.. In CTAB/n-hexanol/hexane reverse micelles, Subtilisin Carlsberg exhibited a mini-increase in α-helix content and an increase in β-sheet content. It could be concluded that both AOT and CTAB reverse micelles induced a more regular secondary structure.The

different influence of reverse micelles on the secondary structure of α-chymotrypsin and Subtilisin Carlsberg was contributed to the difference of protein flexilbility

1. Introduction

Reverse micelles are spherical water droplet surrounded by a monolayer of closely packed surfactant molecules dispersed in apolar solvent[1]. Reverse micelles have been attempted for use in separation and purification of bioproducts and noticed for its energy-saving feature and the possibility of sequential operations[2].

Reverse micelles are also used as organic media to perform biocatalysis[3]. Compared with ordinary organic media, reverse micelles exhibit the advantages of solubilization of both hydrophilic and hydrophobic substrates/products, hindered side reactions, avoided aggregation and easy scale-up[4].

α-chymotrypsin (CMT, E.C.3.4.21.1) and Subtilisin Carlsberg (SUBCARL, E.C.3.4.21.14) are well known serine proteases. They show a high degree of similarity in molecular weight, size of protein molecule, organization of the active site, and substrate specifity [5].

However, the structures, stabilty, and stabilizing forces of the two proteins are significantly differerent. SUBCARL is a single chain polypeptide of 274 amino acid residues, with two Ca^{2+} binding sites and no disulphide bonds. The stabilizing forces are hydrogen bonds and hydrophobic interactions, a weakening of the hydrogen bonds alone by urea or a weakening of the hydrophobic bonds, by its self is insufficient to disrupt the globular structure. SUBCARL is characterized by a pronounced structural lability in acid solutions. Loss of stability occurs even at pH=4[6].

CMT, con

sisting of 241 amino acids, is comprised of three polypeptide chains, which is held together by five disulfide bonds. No Ca^{2+} binding site was found in CMT. CMT could function in the presence of 2M guandidine hydrochloride and 0.1% SDS. It is stable for days in solution at pH=3.0. CMT is least stable in ethanol; the catalytic activity of CMT at an organic solvent of 95% is similar or higher to that in water [7].

Although a considerable volume of work have been published on extraction of proteins by reverse micelles and catalysis of enzyme entrapped in reverse micelles, only a little work has been reported about the effect of reverse micelles on the secondary structure of protein. As to CMT and SUBCARL, with similar molecular weight and active site and different conformation, the compare of the influence of reverse micelles on the secondary structure become very interesting.

Fourier transform infrared spectroscopy (FTIR) has recently become very popular for characterisation of proteins. Now the FTIR and the developed analysis procedures, e.g. Fourier self-deconvolution, second derivation, and curve-fitting have helped to measure the secondary structure of enzyme quantitatively. The method can be used in reverse micelles thanks to the transparent properties of this system. The amide I bands between 1700cm-1~1600cm-1 due to the C=O stretch vibration are the most important part to study the secondary structure of protein [8,9].

In this paper, we have investigated the effects of two kinds of reverse micellar solution, AOT (sodium di [2-ethylhexyl] sulfosuccinate) /isoocatne and CTAB (cetyltrimethylammonium bromide)/hexanol/isooctane, on the secondary structure of CMT and SUBCARL. Furthermore a comparison was carried out between the change of CMT and that of SUBCARL.

2. MATERIALS AND METHODS

2.1 Materials

Bovine pancreatic α-chymotrypsin (MW=25K, pI=8.5) was purchased from Bioreagent Branch,

Shanghai Chemical Reagent company. SUBCARL and AOT were obtained from Sigma and used without further purification. All other reagents were purchased from Beijing Chemical Reagent Company.

2.2 Preparation of samples

Protein solution (5%, w/v) was prepared by dissolving CMT and SUBCARL in D_2O respectively. AOT (50mM)/isooctane reverse micelles (AOT RM) and CTAB (50mM)/15%(v/v) reverse micelles (CTAB RM) n-hexanol/n-hexane were prepared. Protein solutions with concentration of 30% were also prepared, and used for injection into reverse micelles. A clear reverse micellar solution containing protein can be obtained simply by handshaking. The concentration of protein in reverse micellar solution was about 0.6% (w/v). The water content W_0 ([H_2O]/[AOT] mole ratio) was about 22. All solution of protein in D_2O were prepared 48h before using in order to make sure the H-D exchange was completed.

2.3 Measurement of infrared spectra and data manipulation

Infrared spectra was measured with a VECTOR22 (Bruker company, germany) Fourier transform infrared spectrophotometer at 20℃. Samples were placed in a BaF2 cell. For each spectrum, 64 interferograms were collected with a 2 cm-1 resolution. Reference spectra were recorded under identical conditions with media containing no protein.

All data manipulation was carried out using OPUS 4.2.Firstly, the spectra of D2O and reverse micellar solution was subtracted from the observed protein spectra according to the criteria of giving a straight baseline between 2000~1800 cm-1. The signal-to-noise of these spectra was high enough to perform subtraction. Then the amide I spectral region (1700cm-1~1600cm-1) was cutted down from the subtracted spectra. Secondly, all spectra were analyzed by second derivation the amide I for their component composition. Second derivative spectra were smoothed with 11 points function. Thirdly, all spectra were resolution-enhanced by Fourier Self-Deconvolution (FSD) prior curve fitting. The parameters chosen (full width at half-maximum = 18cm-1, the enhancement factor k=2.0) were chosen in the range of those published for other proteins. The conservative values chosen for FSD eliminate the risk of over deconvolution. Finally, Gauss curve fitting was carried out. The frequencies of the band centers determined from second derivative spectra were used as starting parameters and the line shape was a mixture of Lorentz and Gauss. Levenberg-Marquardt algorithm was selected, and the residual mean square error was lower than 0.0002.The individual bands were assigned to kinds of secondary structures, and the fractional areas gives the fraction of secondary structure elements.

3 RESULTS AND DISCUSSION

3.1 FTIR spectra characterization and band assignment of CMT

FSD spectra of CMT dissolved in D_2O, AOT RM and CTAB RM and the corresponding bands resulted from curve fitting are depicted in Fig.1, Fig.2, and Fig.3 respectively. The bands at around 1651 cm^{-1}, which are the band at 1652 cm^{-1} in Fig.1 and that at 1651 cm^{-1} in Fig.2, are definitely due to α-helix, according to the reports of Chang [10]. But the band shifts to 1654 cm-1 in CTAB reverse micelles, which is assigned to α-helix because there is only one band in amide I region for α-helix with frequency around 1650-1660 cm-1for most of the proteins. [9] The shift may indicate some minor rearrangements within the α-helix secondary structure . This interpretation is supported by a wide variety of frequencies where α-helix absorb in various proteins likely indicating only subtle differences in hydrogen bond.

Three bands at 1629, 1636, 1673 cm-1 in Fig.1, those at 1632, 1639, 1674 cm-1 in Fig.2, and those at 1625, 1636, 1676 cm-1 in Fig.3 could be assigned to β-sheet structure. This was strongly supported by previous infrared studies on CMT by Thomas [9].

The band at 1643 cm-1 in Fig.1, that at 1645 cm-1 in Fig.2 and that at 1646 cm-1 in Fig.3 were

assigned to irregular structure according to the reports of Thomas [9]. The bands at 1657, 1665, 1679, 1688 cm-1 in Fig.1, those at 1658, 1667, 1681, 1688 cm-1 in Fig. 2 and those at 1662,1670, 1682 and1685 cm-1 in Fig.3 were assigned to turn structure [9].

3.2 General features and band assignment for SUBCARL

Figures 4, 5 and 6 showed the FSD spectra of SUBCARL and the corresponding bands resulted from curve fitting in D_2O, AOT RM, and CTAB RM respectively. Because of discrepancies with peak assignments, we had tentatively assigned our secondary structure elements to agree with Griebenow and Xu [11,12] noting of cause that these assignments can change in the future.

Generally there is only one band for α-helix in the region of amide I. SUBCARL seems to contain two bands for a-helical structure, which was attributed to some heterogeneity in its a-helix composition. So the bands at 1658, 1666 cm^{-1} in Fig.4, those at 1657, 1665 cm^{-1} in Fig.5, and those at 1658, 1666 cm^{-1} in Fig.6 are assigned to α-helix.

The bands at 1631,1687 cm^{-1} in Fig.4, and those at 1631,1687 cm^{-1} in Fig.5, and those at 1631,1690 cm^{-1} in Fig.6 were attributed to β-sheet structure. The bands at 1674,1680 cm^{-1} in Fig.4, those at 1673,1678 cm^{-1} in Fig.5, those at 1674,1680 cm^{-1} in Fig.6 were attributed to β-turn structure. The band at 1645 cm^{-1} in Fig.4 and Fig.5, and that at 1646 cm^{-1} in Fig.6 were attributed to irregular structure. The band at 1617 cm^{-1} in Fig.4, that at 1618 cm^{-1} in Fig.5, and that at 1615 cm^{-1} in Fig.6 were considered to arise from amino acid side-chain vibration [11].

Band shift could be found when SUBCARL was in AOT RM or CTAB RM, which implied the arrangements of secondary structure. These arrangements could be attributed to the abnormal environment of reverse micelles.

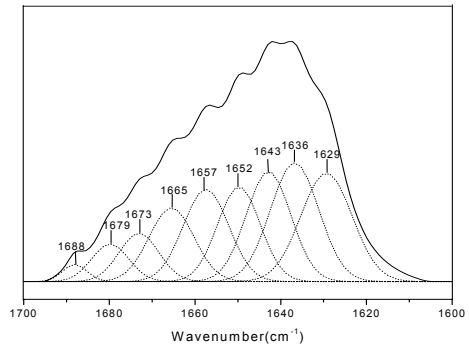

Fig.1. Fourier self-deconvolved FT-IR spectra (solid line) and the individual bands (dotted line) of α-chymotrypsin dissolved in D_2O.

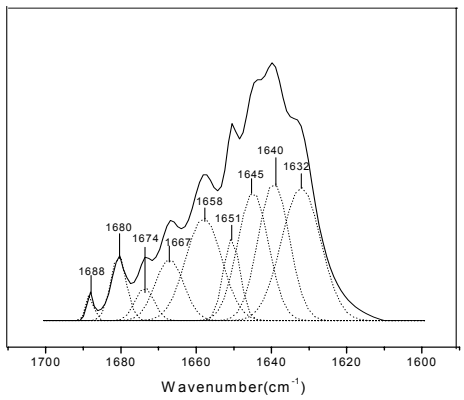

Fig.2. Fourier self-deconvolved FT-IR spectra (solid line) and the individual bands (dotted line) of α-chymotrypsin dissolved in AOT reverse micelles.

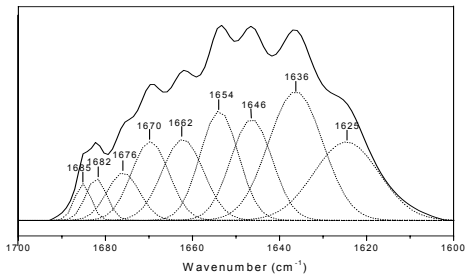

Fig.3. Fourier self-deconvolved FT-IR spectra (solid line) and the individual bands (dotted line) of α-chymotrypsin dissolved in CTAB reverse micelles.

3.3 Quantification of protein secondary structure

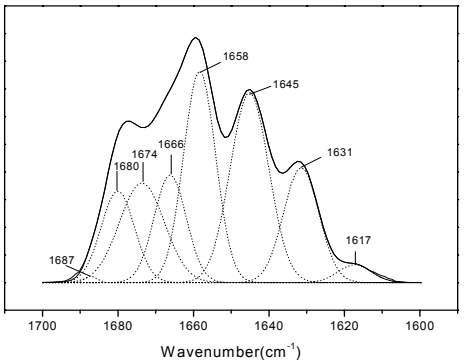

Fig4. Fourier self-deconvolved FT-IR spectra (solid line) and the individual bands (dotted line) of Subtilisin dissolved in D_2O.

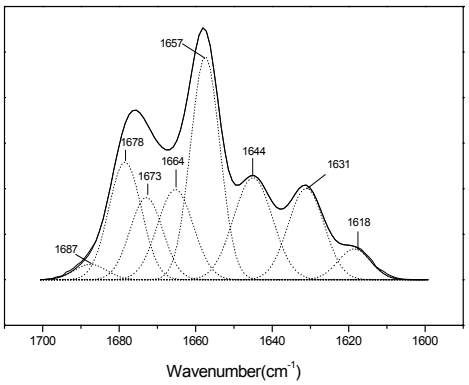

Fig5. Fourier self-deconvolved FT-IR spectra (solid line) and the individual bands (dotted line) of Subtilisin dissolved in AOT/isooctane reverse micelles.

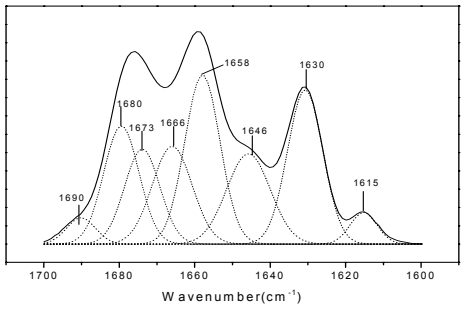

Fig.6. Fourier self-deconvolved FT-IR spectra (solid line) and the individual bands (dotted line) of Subtilisindissolved in CTAB-hexanol-hexane reverse micelles.

The areas of assigned amide I bands resulted from curve fitting are corresponding linearly to the amount of different types of secondary structure in protein. This procedure provided a very good estimate of protein secondary structure and has been adopted by others with some modifications [9]. The method of curve fitting analysis was used to determine the secondary structure of CMT and SUBCARL in D_2O, AOT RM and CTAB RM. The results were shown in Tab.1 and Tab.3 respectively. The calculated results of percentage of α-helix, β-sheet, β-turn and irregular structure are listed in Table 2 and Table 4.

CMT is a primarily β-sheet protein. As shown in Table 2, the content of α-helix, β-sheet, β-turn and irregular structure in D_2O were 9%, 48%, 30% and 13% respectively, which were in excellent agreement with X-ray structural data and the IR-SD (infrared spectra-secondary derivative) analysis result of Dong et al.[13].

By comparing with the structure of CMT in D_2O, the secondary structure in AOT RM and CTAB RM showed great difference. CMT showed a decrease in α-helix content from 9% to 6.2% and a decrease in β-sheet content from 48 to 45.9% in AOT/isooctane reverse micelles which implied a more loose and irregular structure. This was supported by the inactivation of CMT in AOT reverse micelles reported by Marzola [14]. But CMT showed an increase in α-helix content from 9% to 13.83% and a mini decrease in β-sheet content from 48% to 46.7%, which implied a more regular structure. Caldararu reported that the conformation of CMT in CTAB reverse micelles showed more activity than that in AOT reverse micelles [15]. This was in agreement with the changes of secondary structure as mentioned above.

SUBCARL is a protein with more structure of a-helix. As shown in Table 4, the content of α-helix, β-sheet, β-turn and irregular structure in D_2O were 34.4%, 14%, 27.3% and 24.5% respectively, which were in excellent agreement with that calculated from the X-ray struture[16] and the report of Griebenow [11].

SUBCARL showed an increase in α-helix content from 34% to 42% and an increase in β-sheet content from 14% to 15.1% in AOT/isooctane reverse micelles, which implied a more regular structure. AOT/isooctane was reported to exhibit an inhibitory action towards SUBCARL [17]. This meant that the structural rearrangement caused a decrease of activity. In CTAB/n-hexanol/hexane reverse micelles, SUBCARL exhibited a mini-increase in α-helix content from 34% to 34.7% and an increase in β-sheet content from 14% to 21.9%. It could be concluded that both AOT and CTAB reverse micelles induced a more regular secondary structure.

What factor induces the difference of the change of secondary structure between CMT and SUBCARL in reverse micelles? Does this relate to the difference of protein structure? The stability of a globular protein could be quantified by the data of thermodynamics between the native state and the conformation-changed state. The temperature and enthalpy of conformation transition of CMT was reported to be 60 ^{0}C and 710 KJ/mol [18], while those of SUBCARL were of 58.5 ^{0}C and 370 KJ/mol [19], which is lower than CMT. This means that the stability of SUBCAR is lower than the stability of CMT, in another word, the conformation flexibility of SUBCARL is higher than CMT.

In the special environment of reverse micelles, SUBCARL molecule is more flexible, and could change its structure easily to adapt to the environment, this induces a more regular structure in reverse micelles. CMT molecule is more rigid, less adaptive to the environment, but the environment of reverse micelles affected the protein structure, change it locally, which causes a more loose structure in AOT reverse micelles. The more regular structure of CMT in CTAB RM probably was caused by the interaction between CTAB molecule and CMT protein molecule.

Table 1. Infrared band positions, band areas determined by curve fitting, and band assignments in the amide I spectral region of α-chymotrypsin

State	Band position[a] (cm^{-1})	Area(%)[a]	Assignment
CMT in D_2O	1629±0	20.6±2	β sheet
	1636±0	19.4±1	β sheet
	1643±1	13.1±2	irregular [b]
	1652±1	9±1	A helix
	1657±2	13.4±2	B turn
	1665±1	10.9±1	B turn
	1673±2	8.0±1	B sheet
	1679±0	4.4±2	B turn
	1688±0	1.3±1	B turn
CMT in AOT RM	1632±1	23.7±1	B sheet
	1639±2	19.4±3	B sheet
	1645±0	17.9±2	irregular [b]
	1651±1	6.2±1	A helix
	1658±2	16.8±1	B turn
	1667±1	7.9±1	B turn
	1674±2	2.8±1	B sheet
	1681±0	4.4±1	B turn
	1688±0	0.9±1	B turn
CMT in CTAB RM	1625±0	17.6±2	B sheet
	1636±0	24.1±1	B sheet
	1646±0	13.93±3	Irregular [b]
	1654±1	13.83±4	A helix
	1662±1	11.2±2	B turn
	1670±1	9.7±1	B turn
	1676±2	4.99±2	B sheet
	1682±0	2.76±1	B turn
	1685±0	1.86±1	B turn

[a] The ± values are standar deviations calculated by analyzing three individual spectra in each case.

[b] Irregular structures included random coils and extended chains.

Table 2 Secondary structure of α-chymotrypsin in D_2O and reverse micelles

state	Secondary structure			
	α-helix	β-sheet	β-turn	irregular
In D_2O	9±1	48±3	30±2	13±1
In AOT RM	6.2±1	45.9±4	30.0±4	17.9±2
In CTAB RM	13.83±1	46.7±4	25.52±4	13.93±1

Table3. Infrared band positions, band areas determined by curve fitting, and band assignments in the amide I spectral region of Subtilisin Carlsberg

State	Band position(cm^{-1}) [a]	Area(%)[a]	Assignment
SUB in D_2O	1617±0	2±1	side chain
	1631±0	13.6±2	B sheet
	1645±1	24.5±3	irregular [b]
	1658±2	22.8±1	α helix
	1666±1	11.6±2	α helix
	1674±2	15.0±1	β turn
	1680±0	10.3±2	β turn
	1687±0	0.3±1	β sheet
SUB in AOT RM	1618±0	4.0±1	side chain
	1631±0	13.1±1	β sheet
	1645±1	15.9±4	irregular [b]
	1657±3	26.1±2	α helix
	1665±2	12.9±1	α helix
	1673±1	10.7±1	β turn
	1678±0	15.3±1	β turn
	1687±2	2.0±2	β sheet
Sub in CTAB RM	1615±2	3±2	side chain
	1631±2	19.21	β sheet
	1646±0	13.88±3	irregular [b]
	1658±1	21.03±2	α helix
	1666±1	13.73±1	α helix
	1674±0	11.82±1	β turn
	1680±1	14.71±2	β turn
	1690±1	2.63±1	β sheet

[a] The ± values are standard deviations calculated by analyzing three individual spectra in each case.

[b] Irregular structures included random coils and extended chains.

Table 4 Secondary structure of Subtilisin Carlsberg in D_2O and reverse micelles

state	Secondary structure			
	α -helix	β -sheet	β -turn	irregular
In D_2O	34.4±2	14±2	25.2 ±3	24.5±3
In AOT RM	39±2	15.2±3	26.0 ±2	15.9±3
In CTAB RM	34.7±3	21.9±3	26.53 ±2	13.88±3

4. Conclusions

In this paper, we took α -chymotrypsin and Subtilisin Carlsberg as model proteins to investigate the effect of reverse micelles (AOT /isooctane and CTAB/ n-hexanol /hexane) on the secondary structure of

proteins by FTIR spectroscopy.

Both α-chymotrypsin and Subtilisin Carlsberg showed the spectral changes in the amide I which reflected the structural changes in AOT/isooctane and CTAB/n-hexanol/hexane reverse micelles. The secondary structural elements were quantified from the individual areas of Gauss bands. α-chymotrypsin showed a decrease in α-helix content and a decrease in β-sheet content in AOT/isooctane reverse micelles which implied a more loose and irregular structure. α-Chymotrypsin showed an increase in α-helix content and a mini decrease in β-sheet content, which implied a more regular structure. Subtilisin Carlsberg showed an increase in α-helix content and an increase in β-sheet content in AOT/isooctane reverse micelles, which implied a more regular structure. In CTAB/n-hexanol/hexane reverse micelles, Subtilisin Carlsberg exhibited a mini-increase in α-helix content and an increase in β-sheet content. It could be concluded that both AOT and CTAB reverse micelles induced a more regular secondary structure.The different influence of reverse micelles on the secondary structure of α-chymotrypsin and Subtilisin Carlsberg was attributed to the difference of protein flexilbility

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