Two-Dimensional Infrared, Two-Dimensional Raman, and Two-Dimensional Infrared and Raman Heterospectral Correlation Studies of Secondary Structure of β-Lactoglobulin in Buffer Solutions

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Attenuated total reflection (ATR)/infrared (IR) and Raman spectra were measured at room temperature for β-lactoglobulin (BLG) in phosphate buffer (pH 6.6) solutions over a concentration range of 1−5 wt %. Two-dimensional (2D) IR and 2D Raman correlation spectra in the amide III region were generated from the concentration-dependent spectral variations of the BLG solutions to investigate band assignments in the region and to explore concentration-induced conformational changes in BLG. The great resolution enhancement yielded by the 2D IR and 2D Raman spectra enabled us to propose very detailed band assignments for the amide III region. Moreover, the basis of the sign of the asynchronous cross-peaks, we revealed the sequence order of the secondary structure changes induced by the protein association; the changes in the random coil structure exposed to water occur first, and then those in other secondary structure elements follow. 2D IR−Raman heterospectral analysis was also attempted for the same IR and Raman data. The heterospectral correlation maps elucidated the correlation between IR and Raman bands in the amide III region, confirming their band assignments.

Introduction

Generalized two-dimensional (2D) correlation spectroscopy,[1−3] which is an extension of the original 2D correlation spectroscopy,[4] has recently been applied extensively to protein research through studies on secondary structure, denaturation, protein unfolding, and hydration.[5−15] The new method allows one to employ a variety of perturbations to generate 2D correlation spectra of proteins, including not only time but also any other physical variables such as temperature, pressure, concentration, and composition.[1,2] By use of 2D correlation spectroscopy, it is possible to enhance the resolution of individual component bands in the vibrational spectra of proteins. Moreover, this method permits one to probe a series of events occurring during denaturation, adsorption, formation of protein aggregates, hydrogen−deuterium exchange, and so on. Thus far, however, all of the 2D correlation studies of proteins have been concerned with infrared (IR) or near-infrared (NIR) spectroscopy, and there is no 2D Raman study of proteins.[5−15] In addition, most of the 2D IR studies focus on the amide I band region.

The purpose of the present study is to apply 2D correlation spectroscopy to the concentration-dependent Raman spectral variations of β-lactoglobulin (BLG) in buffer solutions and to explore the possibility of IR−Raman heterospectral analysis for protein research. This study is strongly related to the study reported in the preceding paper,[15] in which we discussed 2D attenuated total reflection (ATR)/IR correlation spectra of adsorbtion-induced and concentration-dependent spectral variations of BLG in aqueous solutions. We focused solely on the amide I region in the preceding paper.

The idea of making a correlation between the two kinds of spectroscopy is not new, but it was very difficult to achieve. Generalized 2D correlation spectroscopy has enabled one to try it quite easily.[1,2] Heterospectral analyses were attempted for the 2D IR−Raman analysis of *N*-methylacetamide,[16] the 2D IR−NIR study of nylon-12,[17] and the 2D IR−NIR investigation of ribonuclease A.[9] However, the potential of 2D heterospectral analysis has not yet been well investigated.

Recent rapid developments in FT-IR spectroscopy have enhanced the usefulness of IR spectroscopy for the protein research in aqueous solutions.[18−22] Most often, the IR analysis of protein secondary structures is based on the amide I region because the amide I band is strong and the structure−spectra correlation is most well-established for this region. In the case of Raman spectra, both amide I and amide III regions are used for estimating the secondary structure elements in proteins.[20,23−29]

Although the relationships between the vibrational frequencies of the amide bands and the conformations of peptide backbones have been explored extensively through both experiments and theoretical calculations, the IR and Raman studies of proteins still suffer from two common problems.[20−22,26−29] One is interference with a water band near 1650 cm^{-1} that covers amide I bands. Thus, one must subtract a spectrum of water (or buffer solution) from a spectrum of protein solution. A number of methods for subtracting the water spectrum have been proposed,[30,31] but none of them is perfect. Another problem is severe overlapping of many component bands in the amide I, amide II, and amide III regions.

In the present study, 2D correlation analysis has been applied to ATR/IR and Raman spectral data of BLG in buffer solutions to investigate its secondary structure elements. We have focused on the amide III region, 1350−1200 cm^{-1}. This range is free

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from the water interference in both IR and Raman spectra, and the characteristic spectral features, which provide information about the protein secondary structure, are better resolved. Because of these two facts the benefits of the amide III region are substantial.

A discussion of the ATR/IR and Raman spectra in the amide III region may be facilitated by inspection of the structure of BLG elucidated by X-ray crystallography.[32] In short, the secondary structure of BLG mainly consists of antiparallel β-sheets. The core of the BLG molecule is made up of a short α-helix segment and eight strands of antiparallel β-sheet, which wrap around to form a calyx. In solutions, even at room-temperature, BLG undergoes changes in oligomerization at a high concentration, varying its physical and/or chemical properties slightly.[33,34]

In the preceding paper the 2D IR correlation spectra in the amide I region of the concentration-dependent structural changes in BLG have been reported. In the present paper, the amide III regions of the ATR/IR and Raman spectra will be employed for the same purpose.

Background

The basic theory of 2D heterospectral correlation analysis was described in ref 16.

Experimental Section

Samples. β-lactoglobulin (BLG) and phosphate buffer solutions employed were the same as those reported in the preceding paper.[15] For IR and Raman measurements phosphate buffer (pH 6.6) solutions of BLG with different concentrations (1, 2, 3, 4, and 5 wt %) were prepared.

Spectroscopy. The IR spectra were measured at 1 cm^{-1} resolution with a Magna 760 FTIR/NIR spectrometer equipped with an MCT detector. To ensure a high signal-to-noise ratio, 512 scans were co-added. A ZnSe horizontal ATR prism with 45° end faces (Spectra-Tech, Inc.) was used in this study. The Raman spectra were measured at 4 cm^{-1} resolution with a JASCO NRS-2100 Raman system equipped with a liquid-nitrogen-cooled CCD detector (LN/CCD-1100PBUVAR, Princeton Instruments). The 514.5-nm line from an argon ion laser (Spectra-Physics 2016) was used as an excitation source for the Raman spectra.

2D Correlation Spectroscopy. The pretreatment of ATR/IR spectra of proteins for 2D correlation spectroscopy was described in detail in the preceding paper.[15] Thus, we describe it briefly here. The IR spectra were first ATR corrected. Next, the IR or Raman spectrum of the buffer solution was subtracted from the IR or Raman spectra of the BLG solution, respectively. Then, baseline correction, maximum entropy smoothing, and normalization over the concentration were performed for the obtained IR and Raman spectra before the 2D correlation spectra were constructed. The 2D software used was the same as that described in the preceding paper.[15]

Results and Discussion

IR and Raman Spectra of β-Lactoglobulin in Buffer Solutions. ATR/IR and Raman spectra in the amide III region, 1330−1200 cm^{-1}, of BLG buffer solutions of 1, 2, 3, 4, and 5 wt % are shown in Figures 1 and 2, respectively. In Figure 1, four bands can be identified for the five spectra: a strong band near 1313 cm^{-1}, a weak band near 1285 cm^{-1}, and two bands at 1258 and 1245 cm^{-1}. In contrast, in Figure 2, five bands can be observed: three at 1319, 1292, and 1277 cm^{-1} with similar

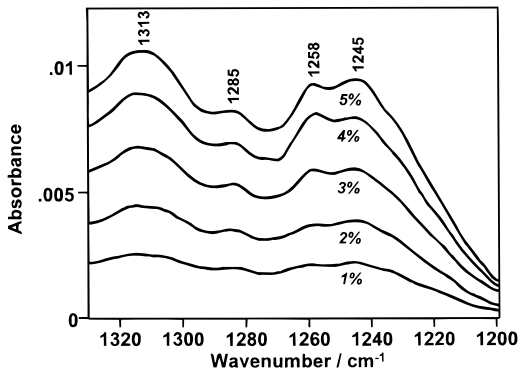

Figure 1. IR spectra in the amide III region of β-lactoglobulin in buffer solutions (1, 2, 3, 4, and 5 wt %).

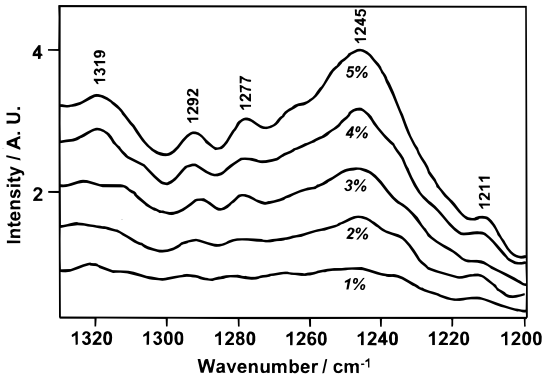

Figure 2. Raman spectra in the amide III region of β-lactoglobulin in buffer solutions (1, 2, 3, 4, and 5 wt %).

intensities, a more intense band at 1245 cm^{-1}, and a very weak shoulder near 1211 cm^{-1}. Band assignments in the amide III region have been investigated extensively for both IR[35−40] and Raman spectrosocopy.[23−29] On the basis of the literature data, we have made band assignments for the IR and Raman spectra of BLG buffer solutions, as presented in Table 1. It is noted that, except for the one band at 1245 cm^{-1}, the positions of the remaining IR and Raman bands are different from each other. The amide III regions of IR and Raman spectra have been used independently to estimate secondary structure elements of proteins.[23−29,35−40] The prediction can be improved by 2D heterospectral correlation that combines simultaneously the IR and Raman data.

2D IR Correlation and 2D Raman Correlation Spectra of β-Lactoglobulin in Buffer Solutions. Figure 3a shows the synchronous 2D IR correlation spectrum constructed from the concentration-dependent spectral changes of BLG buffer solutions. The power spectrum along the diagonal line in the synchronous spectrum also is shown in Figure 3b. Figure 4 depicts (a) the corresponding 2D Raman correlation spectrum, together with (b) the power spectrum. The 2D correlation spectra include contributions from both adsorption-dependent and concentration-dependent spectral variations. On the basis of the preceding paper, in which we revealed that the intensity changes created in the amide I region by adsorption-dependent processes are much less significant than those created by concentration changes,[15] the adsorption-dependent intensity variations can be neglected. In other words, the total intensity changes are dominated by the concentration-dependent processes. The same conclusion can also be reached for the amide III region.

The power spectrum in Figure 3b, reflecting the extent of the intensity variations in IR spectra, yields bands at 1291, 1281, 1277, 1257, 1239, 1227, 1219, and 1207 cm^{-1}. The power

TABLE 1: Amide III Band Positions Observed in 1D Averaged and 2D Correlation IR and Raman Spectra and Their Assignments

	IR			Raman		
	1D spectra	2D spectra		1D spectra	2D spectra	
band assignments	averaged ν (cm^{-1})	synchronous ν (cm^{-1})	asynchronous ν (cm^{-1})	averaged ν (cm^{-1})	synchronous ν (cm^{-1})	asynchronous ν (cm^{-1})
α-helix	1313	1311, 1304	1314, 1305	1319	1318	1311
β-turn, 3_{10}-helix	1285	1291, 1281, 1277	1290, 1278, 1265	1292, 1277	1294, 1282, 1276,	1298, 1283, 1268
random coil	1258	1257	1258	1245	1248, 1243	1255, 1245
β-sheet	1245	1239, 1227, 1219	1243, 1232, 1221		1228	1225
TYR		1207	1211	1211	1212	1214
α-helix	IR	1313−1304	Raman	1319−1311		
β-turn, 3_{10}-helix		1291−1265		1298−1265		
random coil		1258−1257		1255−1243		
β-sheet		1245−1219		1235−1228		
TYR		1211−1207		1214−1211		

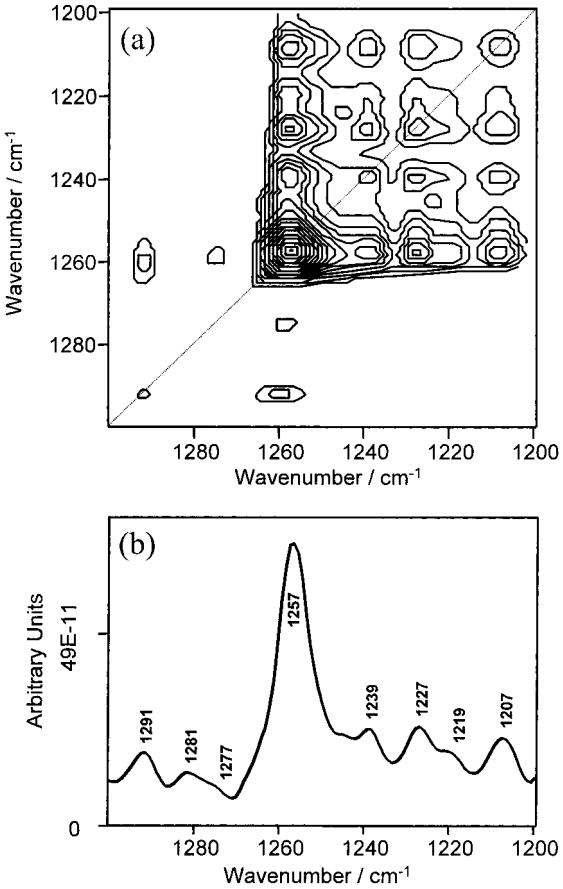

Figure 3. (a) Synchronous 2D IR correlation spectrum generated from concentration-dependent spectral variations of β-lactoglobulin in buffer solutions. (b) Power spectrum along the diagonal line in the synchronous spectrum.

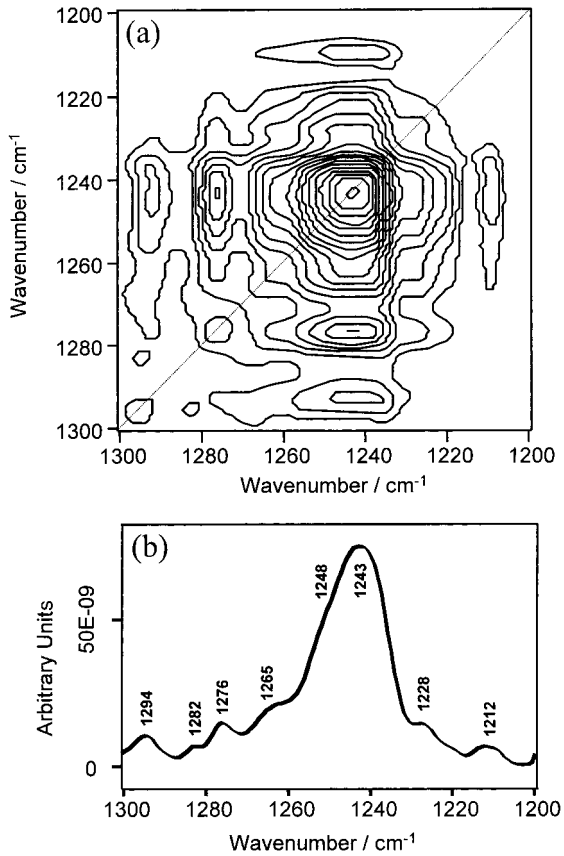

Figure 4. (a) Synchronous 2D Raman correlation spectrum generated from concentration-dependent spectral variations of β-lactoglobulin in buffer solutions. (b) Power spectrum along the diagonal line in the synchronous spectrum.

spectrum in Figure 4b characterizesthis extent in the Raman spectra by developing bands at 1294, 1282, 1276, 1265, 1248, 1243, 1228, and 1212 cm^{-1}. Particularly striking in the power spectra is that the infrared band at 1257 cm^{-1} and the Raman band at 1243 cm^{-1} show much greater intensity variations compared with the other bands.

The concentration change in the BLG solutions introduces subtle differences in the environment of BLG, leading to the association of proteins.[33,34] Our 2D IR study of the amide I region of BLG reported in the preceding paper revealed that the secondary structure and the hydration of BLG are changed significantly by the concentration.[15] It is very likely that exposed residues of BLG are responsible for these concentration-dependent structural variations. Water molecules can reform some of the hydrogen bonds in the backbone chains and also in the side chains located on the exterior of the protein molecule. The observations in the power spectra suggest that the unordered component of BLG is affected most strongly with the association of the protein.

Figure 5a and b shows an asynchronous 2D IR correlation spectrum constructed from the concentration-dependent spectral variations of BLG buffer solutions and a slice spectrum at 1258 cm^{-1}, respectively. The asynchronous spectrum shows two very interesting results. One is that the band at 1258 cm^{-1}, which is due to the unordered forms, shares cross-peaks with a number of bands attributed to β-turns (1290 cm^{-1}), α-helixes (1278 cm^{-1}), and β-sheet structures (1243 and 1233 cm^{-1}). The other is that the band at 1265 cm^{-1} shares cross-peaks with the bands at 1238, 1227, and 1208 cm^{-1}. This band can be assigned to

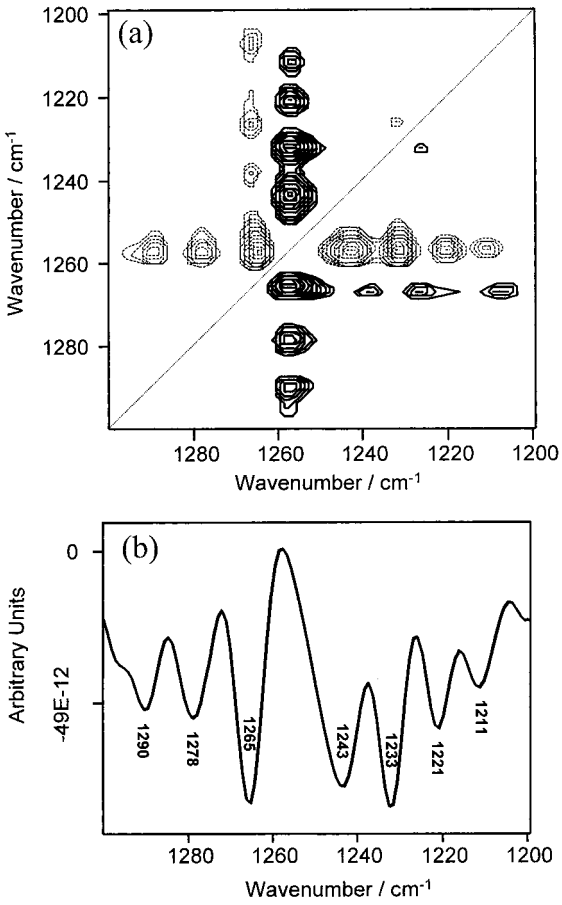

Figure 5. (a) Asynchronous 2D IR correlation spectrum constructed from the concentration-dependent spectral variations of β-lactoglobulin in buffer solutions. (b) Slice spectrum at 1258 cm^{-1}.

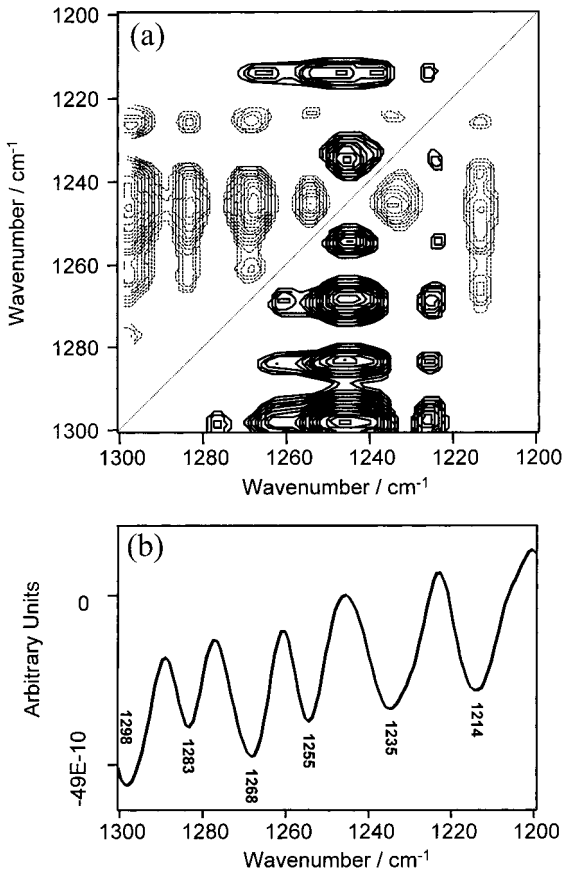

Figure 6. (a) Asynchronous 2D Raman correlation spectrum constructed from the concentration-dependent spectral variations of β-lactoglobulin in buffer solutions. (b) Slice spectrum at 1245 cm^{-1}.

exposed tyrosine (Tyr) residues of BLG.[38] According to the rule proposed by Noda,[1] the signs of the asynchronous cross-peaks show the following sequence of the spectral events occurring during the concentration change:

$$(1258) \rightarrow (1238, 1227) \rightarrow (1290, 1278, 1243, 1232, 1221, 1211) \rightarrow (1265)\ cm^{-1}$$

The above sequence reveals that even small changes in concentration modify absorption in range assigned to the random coil structures (1258 cm^{-1}), whereas the other range of the amide III vibration remains unaffected. Most likely, the concentration changes are directly associated with the changes in the interaction between the water molecules from first hydration shell and the most hydrophilic part of BLG built from the random coil elements. A further increase in concentration causes the next intensity variations at frequencies assigned to the more "exposed" β-sheets (1238 and 1227 cm^{-1}) and nearly simultaneously changes assigned to β-turns (1290 and 1278 cm^{-1}), and "buried" β-sheets absorbing at (1232 and 1221 cm^{-1}). It seems that, during the hydration changes occurring in the first stage, BLG becomes more expanded and open to water penetration. In that case, interactions between the elements residing in its interior undergo changes, giving rise to the intensity variations. At the last stage of the concentration increase, the band due to the Tyr residues experiences an intensity change. This sequence of intensity variations is consistent with that obtain by the 2D analysis of the amide I region.[15]

Figure 6a and b depicts an asynchronous 2D Raman correlation spectrum constructed from the concentration-dependent spectral variations of BLG buffer solutions and a slice spectrum at 1245 cm^{-1}, respectively. As in the case of the 2D IR asynchronous spectrum, the band at 1245 cm^{-1} due to the unordered elements has cross-peaks with a number of bands. The signs of the 2D Raman asynchronous spectrum suggest the following sequence of the spectral changes:

$$(1245, 1225) \rightarrow (1277, 1261) \rightarrow (1255, 1235) \rightarrow (1283, 1298, 1268) \rightarrow (1214)\ cm^{-1}$$

Again, the above scheme shows that even small concentration changes first affect the structure of random coil (1245 and 1225 cm^{-1}). Then, other secondary structure elements follow with further concentration increases in the same way as was described in the previous section. The bands at 1268 and 1214 cm^{-1} can be assigned to the Tyr residues.[24,26] The intensity changes in the bands due to the Tyr residues occur last in both the IR and the Raman spectra. The present 2D IR and Raman studies indicate that, in BLG, the unordered elements reside on the outer surface of the protein and other elements such as β-turns, β-sheets, and α-helices are less accessible to water molecules. This conclusion is in a good agreement with that obtained by X-ray crystallographic study.[32]

To ascertain the band assignments in the IR and Raman spectra of BLG buffer solutions, we performed a 2D IR−Raman heterospectral analysis. Figure 7a and b shows the 2D IR−Raman heterospectral synchronous and asynchronous correlation maps, respectively. Figure 8 illustrates the slice spectra at 1283 and 1256 cm^{-1} of infrared frequencies in the synchronous spectrum. The slice spectra reveal that there are four Raman bands in the 1255−1235 cm^{-1} region. A positive cross-peak in

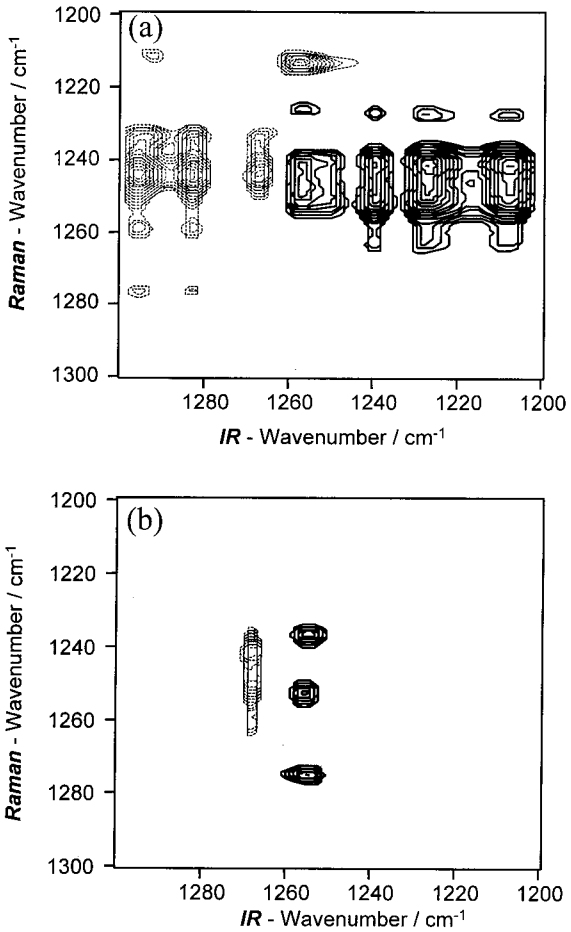

Figure 7. (a) Synchronous and (b) asynchronous 2D IR-Raman heterospectral correlation spectra generated from the concentration-dependent spectral variations of β-lactoglobulin in buffer solutions.

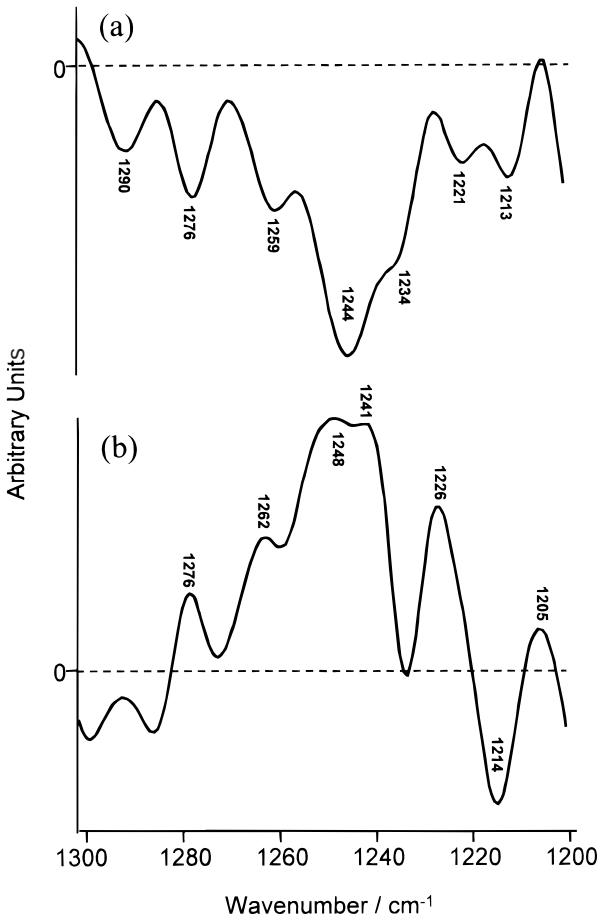

Figure 8. Slice spectra at (a) 1283 and (b) 1256 cm^{-1} of infrared frequencies in the synchronous 2D IR−Raman heterospectral correlation shown in Figure 7a.

the IR−Raman heterospectral synchronous correlation means that two bands sharing the cross-peak have the same origin or change in phase with the concentration, whereas a negative cross-peak means that two bands sharing the cross-peak have different origins. Thus, the negative cross-peaks at (1245, 1295), (1245, 1283), (1245, 1265), (1235, 1295), (1235, 1283), and (1235, 1265) show that the bands at 1245 and 1235 cm^{-1} are assigned to different secondary structure elements from those giving bands at 1295, 1283, and 1265 cm^{-1}. This conclusion is in good agreement with that shown in Table 1. The positive cross-peak at (1245, 1256) cm^{-1} indicates that the Raman band at 1245 cm^{-1} and the IR band at 1256 cm^{-1} arise from the same secondary structure, the unordered form.

The heterospectral asynchronous map shows that the IR component at 1258 cm^{-1} associated with the unordered structures is correlated out-of-phase with the Raman components at 1235 and 1255 cm^{-1} attributed to the β-sheet and the unordered structures, respectively, and with that at 1268 cm^{-1} assigned to the Tyr resides. Probably, the unordered structures giving rise to the IR band at 1258 cm^{-1} and those yielding the Raman band at 1255 cm^{-1} have different origins.

Conclusion

The present study has demonstrated the potential of 2D IR, 2D Raman, and 2D IR−Raman heterospectral correlation analyses in secondary structure studies of proteins based on the amide III region. Both 2D IR and 2D Raman correlation spectra have greatly enhanced spectral resolution in the amide III region of BLG. The existence of a number of component amide III bands overlapping with each other has become clear and even bands due to the amino acid residues can be identified by the 2D correlation analysis. The asynchronous spectra have demonstrated the sequence order of events occurring during the formation of the protein associations. It has been found that the unordered structures accessible to surrounding water change first, and then other secondary structure elements vary. This conclusion is in a good agreement with that reached by our 2D IR study of the amide I region of BLG reported in the preceding paper.[15] The 2D IR−Raman heterospectral correlation analysis has provided new insight into the correlation between the IR and Raman bands in the amide III region and has confirmed some band assignments.

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