Elucidation of Protein−Lipid Interactions in a Lysozyme−Corn Oil System by Fourier Transform Raman Spectroscopy

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Lysozyme (25% in D_2O), corn oil, and their emulsions (10% w/w oil/D_2O solution) were examined by Fourier transform Raman spectroscopy. Emulsions showed three layers, namely, top oil, middle cream, and bottom aqueous layers. Raman spectral analysis revealed hydrophobic interactions involving both protein and lipid components. Compared to lysozyme in D_2O, the difference spectrum obtained after subtraction of oil from the cream layer spectrum showed reduced intensity of tryptophan bands at 760, 1013, 1340, and 1360 cm^{-1}, reduced intensity ratio of the tyrosine doublet at 850 and 830 cm^{-1}, and increased intensity of the C−H bending band at 1455 cm^{-1}. Compared to corn oil, the difference spectrum after subtraction of lysozyme from the cream layer spectrum indicated decreased intensity at 2855 cm^{-1} (lipid CH_2 symmetric stretch) and 3011 cm^{-1} (unsaturated fatty acid hydrocarbon chain =C−H stretch) and a higher intensity ratio of the C−H stretching band at 2900 cm^{-1} to bands at 2885 and 2933 cm^{-1}. Spectra of the top and bottom layers resembled corn oil and lysozyme, respectively, except for changes in the D_2O band. Raman spectroscopy can be used to detect structural changes in proteins, lipids, and D_2O due to protein−lipid interactions.

Keywords: *Raman spectroscopy; lipids; proteins; interactions*

INTRODUCTION

The structure and physical−chemical properties of proteins and lipids influence the formation and stability of emulsions and the texture of many food products. However, the exact mechanism of interactions between the protein and lipid functional groups is not clear.

Raman spectroscopy can provide detailed information on changes in protein and lipid structure (*1*). Unlike many spectroscopic techniques such as circular dichroism and nuclear magnetic resonance (NMR) spectroscopy, Raman spectroscopy is particularly suitable for investigating concentrated and solid samples and, therefore, most food products including gels and emulsions (*2*, *3*).

The application of Raman spectroscopy to food proteins includes studies conducted in our laboratories on the effect of heat processing on α-lactalbumin, *β*-lactoglobulin, and lysozyme (*4*−*6*). These studies showed shifts in the amide I and amide III regions indicating a decrease in the α-helix structure and concomitant increase in the *β*-sheet structure, as well as changes in hydrophobic and disulfide groups. Pioneering studies on protein mixtures of lysozyme with α-lactalbumin and *β*-lactoglobulin undertaken by Howell and Li-Chan (*6*) indicated experimental spectra of protein mixtures that differed from the calculated average spectra of the component individually heated proteins. In addition to electrostatic interactions (*7*), the aggregated complexes showed the involvement of hydrophobic interactions by intensification of spectral bands assigned to CH and CH_2 bending vibrations and a decrease in the intensity of bands assigned to tryptophan residues in a nonpolar environment. Detailed investigations of the CH stretch regions (2800−3000 cm^{-1}) confirmed changes not only in the aromatic and hydrophobic groups but also in those due to polar and hydrophilic amino acids (*8*).

Raman spectroscopy has also been used to characterize lipid molecules. The intensity and location of bands of various fatty acids are affected by the degree of unsaturation and liquid or solid phase (*9*). Acyl chain packing of lipids is reflected in the spectrum, and the relative intensity ratio (I_{2935}/I_{2880}) measures effects originating from changes in intrachain *trans*/*gauche* isomerization superimposed on the chain−chain interactions (*10*, *11*). Polymorphism of diacylglycerols (*12*) and phase transitions of the three crystalline phases (α, *β*, and *γ*) of oleic acid (*13*) have been investigated by Fourier transform (FT) Raman spectroscopy. Changes during the transitions were monitored in various regions of the Raman spectra, including CH stretching, C=O stretching, CH_2 scissoring, C−C stretching, methyl rocking, olefin group, and longitudinal acoustic modes (*13*). In addition to the above, Raman spectroscopy can be used for rapid quantitative analysis of the total degree of unsaturation of lipids using the ratio of the C=C stretching band near 1660 cm^{-1} to the C=O stretching band near 1750 cm^{-1} or the CH_2 scissoring band near 1445 cm^{-1} (*14*−*16*). The relative amounts of saturated, monounsaturated, and polyunsaturated fatty acids can be ascertained using the 3013, 1663, and 1264 cm^{-1} bands (*16*). The ratio of *cis* and *trans* isomers and conjugated products resulting from autoxidation can also be followed using several bands (*17*).

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Although progress has been made in applying Raman spectroscopy to examine protein−protein interactions as described above, few studies have been undertaken on protein−lipid interactions, mostly on biological membrane structures (*18*−*20*). Moreover, many of the studies have focused on changes in the lipid components only, and the contribution of protein components was considered to be insignificant. The region between 100 and 1200 cm^{-1} can give information on the *trans* and *gauche* conformations of the hydrocarbon chains, whereas the 2800−3000 cm^{-1} region relating to C−H stretching is more sensitive to structural changes in lipid−water and lipid−protein−water phase systems (*21*, *22*). In particular, the ratio of the intensity of the peak at 2850 cm^{-1} to that at 2890 cm^{-1} increases with the "looseness" of the lateral packing of disordered hydrocarbon chains. Therefore, the peak at 2850 cm^{-1} is dominant in the liquid state, whereas the peak at 2890 cm^{-1} is more prominent when the hydrocarbon chains are in a crystalline state (*22*). In addition, in the presence of proteins or polypeptides, Larsson and Rand (*21*) suggested that the relative intensity of the 2930 cm^{-1} band increased with increased polarity of the environment around the hydrocarbon chains; thus, changes in this band may indicate whether lipid molecules are associated with proteins.

Despite the increasing information available on individual protein and lipid systems, detailed studies on food emulsions have not been reported. Preliminary investigations of emulsions formed by either vortex action or sonication, using corn oil and lysozyme or dithiothreitol-treated reduced lysozyme, indicated changes in the C−H stretching region suggesting possible hydrophobic protein−lipid interactions and greater ordering of the fatty acid chains (*2*, *23*); these spectral changes were analogous to those observed in the crystalline forms of fatty acids compared to the liquid phase (*9*). Similar conclusions were drawn for studies on emulsions prepared with egg white or β-casein. In the present study, a more detailed investigation of the interaction of lysozyme with corn oil was undertaken using near-infrared (NIR) FT Raman spectroscopy. Difference spectra, obtained by subtracting the individual lysozyme and corn oil spectra from the spectrum of the emulsion, were used to ascertain the groups involved in the interactions. Because the O−H stretching band of water occurs near the C−H stretching region and has a line width in excess of 500 cm^{-1}, samples were prepared as deuterated solutions to minimize the effect of the water signal on the Raman spectra.

MATERIALS AND METHODS

Materials. Deuterium oxide (D-4501, 99.9 atom % D), lysozyme (L-6876, from chicken egg white, 3× crystallized, dialyzed, and lyophilized), and corn oil were from Sigma Chemical Co., Poole, Dorset, U.K.

Methods. To study the interactions between lysozyme [25% (w/v) solutions in D_2O] and corn oil, emulsions consisting of the deuterated solution and 10% (w/w) corn oil were prepared by homogenization using an Omni-mixer homogenizer model 17106 (Waterbury Limited, supplied by Camlab Limited, Cambridge, U.K.). Each 3 mL sample was homogenized at speed 3.5 for 5 min. The emulsions separated into three layers, which were separately analyzed by FT Raman spectroscopy, together with control samples of lysozyme (25% in D_2O) and corn oil on their own.

Raman Spectral Analysis. The Raman scattering of samples placed in 7 mL glass containers (FBG-Anchor, London, U.K.) was measured at ambient temperature on a Perkin-Elmer System 2000 FT Raman spectrophotometer with excitation from an Nd:YAG laser at 1064 nm. Frequency calibration of the instrument was performed using the sulfur line at 217 cm^{-1}. Replicate sets of the samples were prepared and analyzed on two different occasions. For the first set, laser power at the sample was 850 mW and 32 co-added spectra at a resolution of 8 cm^{-1} were obtained for each sample. For the second set, the laser power was 2600 mW and 32 co-added spectra were collected at a resolution of 4 cm^{-1}. Similar results were obtained from both sets of data.

The recorded spectra were analyzed using Grams 32 or Grams 386 software (Galactic Industries Corp., Salem, NH). The cream layer and lysozyme (25% in D_2O) spectra collected at 8 cm^{-1} resolution were baselined and smoothed using Fourier transformation with a degree of smoothing of 65, and the intensity was normalized on the phenylalanine peak at 1004 cm^{-1} (*6*, *8*). The cream layer and lysozyme spectra collected at 4 cm^{-1} resolution were analyzed similarly, except for omission of the smoothing step. Corn oil was normalized on the 2855 cm^{-1} peak. Subtraction of the corn oil spectrum from the cream layer spectrum was performed by zeroing the C═O ester band at 1750 cm^{-1}, which is unique for oil but is absent in proteins; the subtraction of oil was confirmed by the disappearance of the lipid bands near 1300 and 1265 cm^{-1}. The resultant difference spectrum was used to detect any changes in the residual lysozyme signal, by comparison with the normalized spectrum of lysozyme (25% in D_2O) on its own. In addition, subtraction of the normalized spectrum of lysozyme (25% in D_2O) solution from that of the emulsion cream layer was undertaken to give a difference spectrum to detect any changes in the residual oil spectrum. Assignments of the bands in the spectra to protein or oil vibrational modes were made on the basis of the literature (*2*, *16*, *17*, *20*, *24*, *25*) and are shown in Table 1.

Table 1. Assignment of Characteristic Raman Bands in Corn Oil and Lysozyme

wavenumber, cm^{-1}	assignment
	Corn Oil
3011	═CH stretch of ═CHR or $═CH_2$ groups
2960−2850	−CH stretch of *n*-alkyl group
2959	CH_3 antisymmetric stretch
2933	CH_2 antisymmetric stretch
2900	R_3C-H, CH_3 symmetric or CH_2 antisymmetric stretch
2874	CH_3 symmetric stretch
2855	CH_2 symmetric stretch
2730	CH stretch of aliphatic CHO
1750	C═O ester stretch
1660	C═C stretch (*cis*)
1441	CH_2 scissoring
1303	CH_2 in phase twist
1265	═C−H symmetric rock (*cis*)
1083	antisymmetric CCC stretch
973, 916, 874, 844	symmetric CCC stretch, various CCCC stretches
	Lysozyme in D_2O
508	S−S stretching vibrations (*gauche*−*gauche*−*gauche*)
525	S−S stretching vibrations (*gauche*−*gauche*−*trans*)
760	tryptophan
850, 830	tyrosine doublet
936−946	amide III′ helix
965−970	amide III′ random
980−990	amide III′ antiparallel β-sheet
1004	phenylalanine
1013	tryptophan
1062−1080	C−C or C−N skeletal stretch
1250	amide III
1340	CH bending or tryptophan
1360	tryptophan
1455	CH bending
1657	amide I band
2400−2500	D_2O
2800−3100	CH stretch of various amino acids

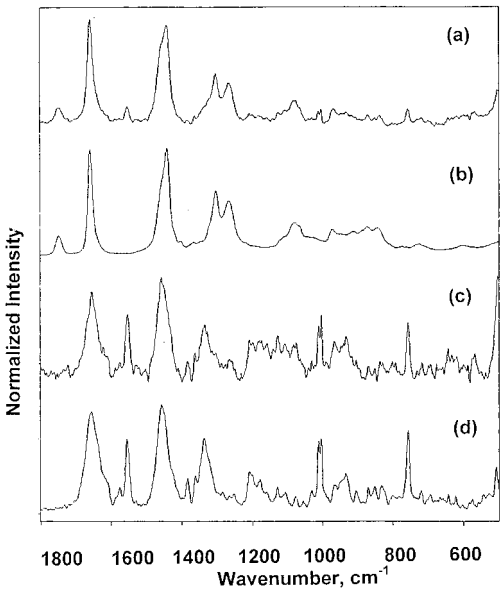

Figure 1. FT Raman spectra in the 500−1800 cm^{-1} region for (a) the cream layer, (b) corn oil, (c) the difference spectrum obtained after subtracting the corn oil spectrum from the cream layer spectrum, and (d) lysozyme in D_2O.

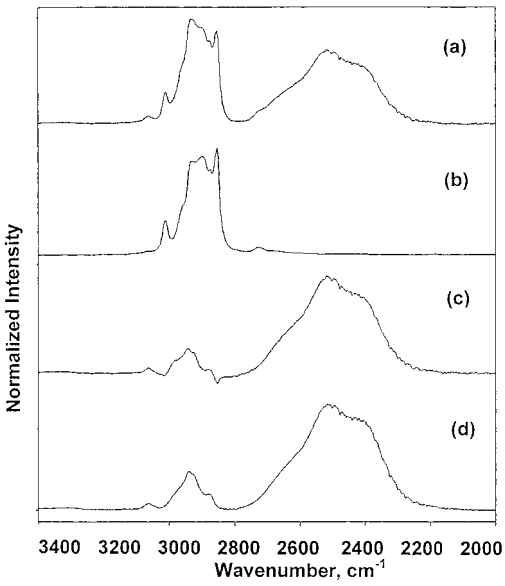

Figure 2. FT Raman spectra in the 2000−3400 cm^{-1} region for (a) the cream layer, (b) corn oil, (c) the difference spectrum obtained after subtracting the corn oil spectrum from the cream layer spectrum and (d) lysozyme in D_2O.

RESULTS AND DISCUSSION

The emulsion separated into three layers, with a top layer of corn oil, a middle cream layer, and an aqueous solution at the bottom of the tube.

Analysis of the Cream Layer. Figure 1a shows the spectrum of the cream layer in the 500−1800 cm^{-1} region. The corn oil spectrum in Figure 1b indicated bands assigned to the following groups: C═O ester stretch at 1750 cm^{-1}; C═C stretch at 1660 cm^{-1}; CH_2 scissoring at 1440 cm^{-1}; CH_2 in phase twist at 1303 cm^{-1} and ═C−H sym rock (*cis*) at 1265 cm^{-1} (*24*). By subtraction of the normalized spectrum of corn oil from the cream layer spectrum, the difference spectrum was obtained (Figure 1c) and compared to the spectrum for lysozyme in D_2O (Figure 1d). If there are no changes in the protein and oil due to specific interactions, the difference spectrum would be expected to resemble the spectrum of lysozyme on its own; however, several differences were observed, indicating protein−lipid interactions.

The difference spectrum (Figure 1c) showed a reduction in the intensity of tryptophan bands at 760, 1013, 1340, and 1360 cm^{-1} compared with that of lysozyme in D_2O (Figure 1d), indicating exposure of tryptophan residues, possibly to a more hydrophilic environment, due to denaturation of the protein molecule. Increased intensity was observed in the region corresponding to CH bending, which could be due to changes in the protein (1455 cm^{-1}), lipid (1441 cm^{-1}), or protein−lipid interactions involving C−H groups.

The intensity ratio I_{850}/I_{830} of the tyrosine doublet at 850 and 830 cm^{-1} decreased from a value of 1.0 for lysozyme in D_2O to a value of 0.5 for the protein in the cream layer as calculated in the difference spectrum. Because a lower I_{850}/I_{830} ratio indicates tyrosine residues in a buried environment or those acting as hydrogen donors rather than acceptors (*2*, *25*), these results suggest a more hydrophobic environment for the tyrosine residues of lysozyme in the cream layer than in D_2O.

The predominant band at 508 cm^{-1} for lysozyme in D_2O appeared to be replaced by a band near 525 cm^{-1} in the difference spectrum, indicating a shift in the conformation of the disulfide groups of lysozyme in the cream layer. Detailed analysis of the ratio of intensities in this region, assigned to S−S stretching of cystinyl residues, was difficult due to the steep background of the spectra in this region. However, similar changes have also been observed in heated lysozyme (*6*).

The amide I band near 1660 cm^{-1} cannot be characterized with respect to the protein secondary structure in the cream layer due to possible interference from the 1660 cm^{-1} band of corn oil. However, the appearance of a band at the 980−990 cm^{-1} region was noted, suggestive of an increase in the protein β-sheet structure, whereas higher intensity of the amide III′ band at 965 cm^{-1} indicated a greater proportion of unordered structures. An increase in the β-sheet structure may be due either to interaction of this structure in the protein with the lipid phase or to interaction among adsorbed protein molecules. If the latter mechanism is operative, the β-sheet content may increase with time, as it has been shown that the packing state of adsorbed protein molecules and surface viscosity change with time (*26*); this aspect requires further study.

Figure 2 shows the region from 2000 to 3400 cm^{-1} including the broad D_2O band centered near 2500 cm^{-1} and the CH stretching region at 2800−3100 cm^{-1}. The CH stretching band of the cream layer (Figure 2a) does not appear as a simple addition of the corn oil (Figure 2b) and lysozyme (Figure 2d) spectra. The difference spectrum (Figure 2c) shows negative signals in this region at 2855 and 3011 cm^{-1}, suggesting protein−lipid interactions involving CH groups.

This was confirmed in the difference spectrum (Figure 3c) derived from the subtraction of lysozyme (Figure 3b) from the cream layer (Figure 3a). In the absence of interactions, this difference spectrum should resemble the spectrum of corn oil (Figure 3d). In fact, the difference spectrum showed a decreased signal at 2855 cm^{-1} (lipid CH_2 symmetric stretch) and at 3011 cm^{-1} (the ═C−H stretch of the unsaturated fatty acid hydrocarbon chains). In addition, the intensity ratios I_{2900}/I_{2933} and I_{2900}/I_{2855} of the C−H stretching bands at 2855,

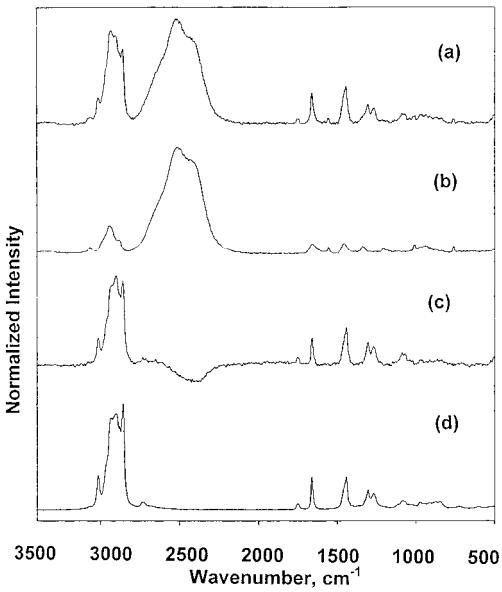

Figure 3. FT Raman spectra in the 500−3400 cm^{-1} region for (a) the cream layer, (b) lysozyme in D_2O, (c) the difference spectrum obtained after subtracting the lyszoyme spectrum from the cream layer spectrum, and (d) corn oil.

2900, and 2933 cm^{-1} are both higher in the difference spectrum than in the spectrum of corn oil alone. These changes are suggestive of hydrophobic interactions involving the C−H groups of the lipid with protein in the cream layer. A similar observation was reported by Larsson (*22*) for the intensity ratio I_{2890}/I_{2850}, which was higher in the case of a milk emulsion than in the separated fat phase. It was suggested that most of the hydrocarbon chains of the milk fat globule membrane and/or those of the triglyceride molecules adjacent to the membrane are crystalline and close packed, and this packing is of importance for emulsion stability. Similarly, a very sharp 2881 cm^{-1} band was assigned to stretching vibrations of ordered chains of oleic acid (*13*), and the location of the predominant peak in the CH stretching region of fatty acids was reported to vary between oleic acid (2850 cm^{-1}), linolenic acid (2930 cm^{-1}), and the trans fatty acid linoelaidic acid (2900 cm^{-1}), reflecting the differences in packing of these fatty acids (*9*). The other bands in the difference spectrum resembled the corn oil spectrum, with the exception of the negative signal in the 2400 cm^{-1} region, which is assigned to vibrational modes of the D_2O molecule.

Analysis of the Bottom Aqueous Layer. Figure 4 shows the spectrum of the bottom aqueous layer compared with that of 25% lysozyme in D_2O solution. The spectra were virtually identical, indicating that the aqueous phase at the bottom of the tube contained protein only. It was interesting to note that the only difference was in the shoulder of the D_2O signal, which was lower for the bottom aqueous layer compared with lysozyme solution. This confirms the changes observed in the D_2O signal of the difference spectrum of the cream layer and corn oil (Figure 3c). It is likely that the vibrational modes of D_2O (Figure 4 inset) would be affected by the presence of corn oil as well as by the protein. In a food emulsion the effect of oil on the water molecules and their subsequent restructuring may also affect adjacent protein groups and hydrophobic interactions.

Analysis of the Top Oil Layer. Figure 5 shows the spectrum of the top layer compared with corn oil on its

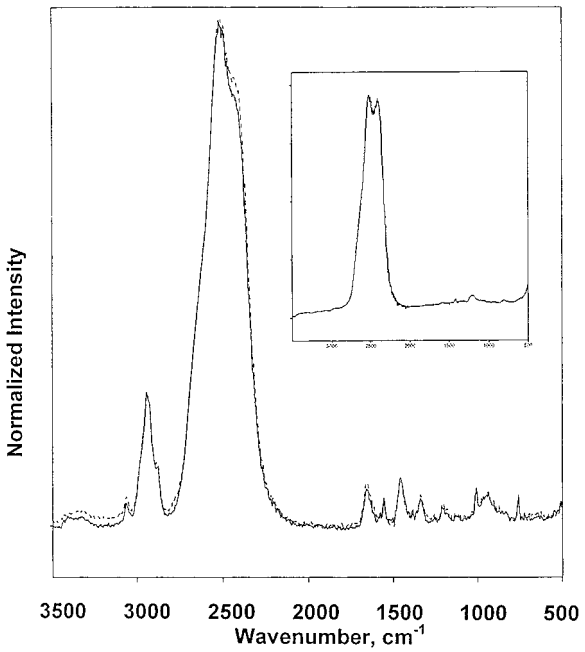

Figure 4. FT Raman spectra in the 500−3400 cm^{-1} region for lysozyme in D_2O (—) compared to the bottom aqueous layer (- - -). The FT Raman spectrum of D_2O alone is shown in the inset.

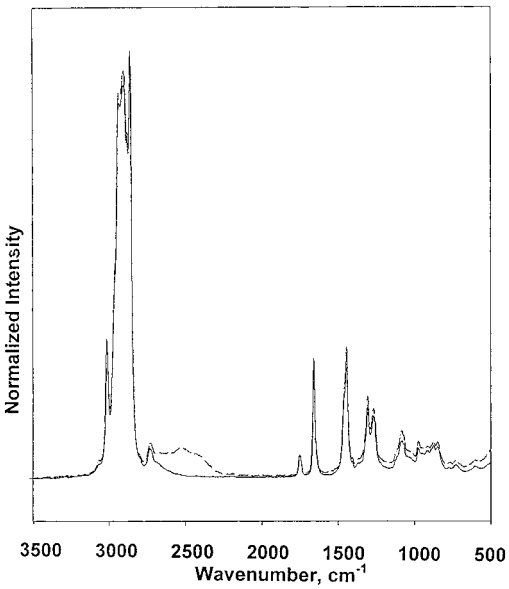

Figure 5. FT Raman spectra in the 500−3400 cm^{-1} region for corn oil (—) compared to the top layer (- - -).

own. The spectra were similar with the exception of the presence of a small D_2O peak in the top layer spectrum, confirming the interaction of lipid with D_2O. Although traces of protein may be present in the top layer and play a role in the lipid−D_2O interface, the low concentrations would not be detectable in the Raman spectrum.

Conclusions. The presence of lipids can alter the molecular structure of proteins and result in changes in exposure of hydrophobic groups, secondary structures, and conformation of disulfide groups. The mechanism of protein denaturation by lipids may be due to protein−lipid complexing or the restructuring of water molecules surrounding proteins. In addition to investigations on structural changes in individual proteins and lipids on processing and storage, Raman spectroscopy

accompanied by detailed spectral analysis can provide a valuable tool for the study of protein−lipid interactions in food emulsions and mixtures as well as in biological membranes and tissues.

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