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Review

Vibrational spectroscopy applied to the study of milk proteins

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Abstract – Vibrational spectroscopy is a versatile tool for analysis of foods including dairy products. This paper provides an overview of techniques that may be useful to study structural properties and interactions of milk proteins. Specific examples of recent research conducted in our laboratory using Raman, micro-Raman and FT-Raman spectroscopy will be described to illustrate their applications for investigating protein-protein, protein-lipid, and protein-polysaccharide interactions, and for elucidating structure-function relationships of whey proteins and peptides. Raman spectroscopy was used to discriminate between structural changes of β-lactoglobulin in transparent fine-stranded versus particulate gels. Formation of particulate gel structures led to a more hydrophilic or exposed microenvironment around tryptophan residues, compared to a more hydrophobic microenvironment in translucent gels. A decrease in the α-helical content was more pronounced in translucent gels, while both types of gels retained considerable β-sheet structures, consistent with the known heat-resistance of the β-barrel structure of β-lactoglobulin. Principal component similarity analysis demonstrated that heat treatment and heat-κ-carrageenan interactions were the most influential factors affecting whey protein structure as monitored by spectral changes. Raman micro-spectroscopy was applied to investigate protein-lipid interactions at the interface between bovine serum albumin solution and mineral or corn oil. While Raman bands assigned to aromatic and aliphatic residues inferred involvement of hydrophobic interactions at the oil-water interface, the secondary structure was not significantly altered. Temperature-dependent changes in the Raman C-H stretching region of liposomes composed of phospholipids with varying headgroups and acyl chains were influenced by the presence of bovine lactoferricin. This approach may be useful to elucidate the interactions of cationic antimicrobial peptides with bacterial versus host cell membranes. A diverse range of other applications as well as new developments in techniques are emerging in the literature, indicating the potential for increasing use of vibrational spectroscopy in the analysis of milk and milk products.

spectroscopy / Raman / protein / structure / interaction

摘要 – 应用振动光谱法研究乳蛋白性质。振动光谱是食品分析中一种通用分析方法。本文论述了振动光谱法研究乳蛋白结构性质和乳蛋白之间的相互作用。近年来本实验室主要采用拉曼光谱法、显微拉曼光谱法和傅里叶变换拉曼光谱 (FT-Raman) 法解释了蛋白-蛋白、脂肪-蛋白及蛋白-多糖之间的相互作用, 并且进一步说明了乳清蛋白和肽的结构与功能之间的关系。拉曼光谱还可以区别 β-乳球蛋白的透明凝胶与其相对应的凝胶粒子之间的结构变化。与具有较多疏水性微环境的透明凝胶相比, 凝胶微粒结构的形成产生了较多亲水基团或者使更多的色氨酸残基暴露在微环境中。在透明凝胶中 α-螺旋含量显著地降低, 而两种凝胶中都保留了大量的 β-折叠结构, 这一点与已知的 β-乳球蛋白的 β-桶结构具有耐热性是一致的。根据对主要化合物的光谱分析证明,

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热处理和热 κ-角叉胶是影响乳清蛋白结构的最重要因素。显微拉曼光谱法用于研究牛血清白蛋白和矿物质或玉米油溶液界面上蛋白-脂肪的相互作用。根据芳香族和脂肪族残基对应的拉曼谱带可以推断在油和水的界面发生了疏水作用,但是次级结构没有发生显著的改变。为了研究乳铁蛋白与磷脂之间的相互作用,测定了不同温度下,由含不同酰基的磷脂组成的脂质体与乳铁蛋白作用的拉曼光谱,这个过程可以用来解释阳离子抗菌肽与细菌和宿主细胞膜之间的相互作用。振动光谱在其他方面的应用已经有过文献报道,尤其应用于乳和乳制品分析的报道逐渐增加。

光谱 / 拉曼 / 蛋白质 / 结构 / 相互作用

Résumé – Spectroscopie vibrationnelle appliquée à l'étude des protéines. La spectroscopie vibrationnelle est un outil adéquat pour l'analyse des aliments, dont des produits laitiers. Cet article présente une revue des techniques qui peuvent être utiles pour étudier les propriétés structurales et les interactions entre protéines laitières. Des exemples spécifiques de recherches récentes conduites dans notre laboratoire utilisant la spectroscopie Raman, micro-Raman et FT-Raman sont décrites pour illustrer leurs applications dans l'étude des interactions protéines/protéines, protéines/lipides et protéines/polysaccharides, et dans l'élucidation des relations structure/fonction des protéines et peptides du lactosérum. La spectroscopie Raman a été utilisée pour faire la distinction entre les changements de structure de gels de β-lactoglobuline filamenteux ou particulaires. La formation de structures de gel particulaire conduisait à un microenvironnement plus hydrophile ou exposé autour des résidus tryptophane comparé à un microenvironnement plus hydrophobe dans les gels translucides. Une diminution du contenu en hélices α était plus prononcée dans les gels translucides, alors que les deux types de gels retenaient une proportion importante de structures en feuillets β, en accord avec la thermorésistance connue de ces structures dans la β-lactoglobuline. L'analyse en composante principale a démontré que les facteurs traitement thermique et traitement thermique + interactions κ-carraghenane étaient ceux qui influaient le plus sur la structure des protéines de lactosérum, comme montré par le suivi des changements de spectre. La spectroscopie micro-Raman a été utilisée pour étudier les interactions sérum-albumine bovine/lipides dans une interface huile-eau. Alors qu'à partir des bandes Raman attribuées aux résidus aromatiques ou aliphatiques on pouvait conclure à l'implication des interactions hydrophobes à l'interface huile-eau, la structure secondaire n'était pas significativement modifiée. Les changements température-dépendants relatifs à la région C-H du spectre Raman de liposomes composés de différents phospholipides étaient influencés par la présence de lactoferricine bovine. Cette approche peut être utile pour élucider les interactions entre peptides antimicrobiens cationiques et bactéries ou membrane cellulaire de l'hôte. Une large gamme d'autres applications ainsi que de nouveaux développements dans les techniques émergent dans la littérature, montrant les potentialités accrues d'usage de la spectroscopie vibrationnelle pour l'analyse du lait et des produits laitiers.

spectroscopie / Raman / protéine / structure / interaction

1. INTRODUCTION

Much research has been conducted in the past few decades to elucidate the structure and function of food proteins, including the proteins in milk and milk products [37]. As a result of their functional properties such as gelling, film-forming, surfactant, emulsifying and foaming properties, proteins play an important role in the textural and sensory properties as well as the stability of food systems. Intra- and inter-molecular interactions of protein molecules are a culmination of intrinsic structural properties of the proteins, and their ability to be involved in disulfide bonds, hydrogen bonding, hydrophobic and electrostatic interactions. These are ultimately influenced by extrinsic factors including constituents in the food, such as other protein molecules, lipids, carbohydrates and even the solvent water, as well as pH, salt or other ions. Furthermore, food processing operations such as

homogenization, heat treatment or drying may also result in changes in protein structure and function.

In order to tailor and control the function of protein components in food, it is important to understand the relationship between the structural properties of proteins and their function. Moreover, relevant information to elucidate this relationship should be obtained by analyzing the proteins as they would typically occur in the food system of interest [19]. In the case of milk and milk products, this may involve relatively high solute concentrations of the proteins in the presence of other ingredients such as fat or oil, sugars, polysaccharides, etc., and may also entail transformation of the proteins from a solution (i.e. fluid milk) to turbid suspensions, solid gels and coagula (e.g. puddings, yogurt, cheese, ice cream) through interactions with the other food components or by processing [9].

Infra-red and Raman spectroscopy are complementary techniques of vibrational spectroscopy that can be applied to analyze protein structure in aqueous solutions as well as in various physical states including films, powders, gels or crystals [19, 20]. In infra-red spectroscopy, the sample absorbs electromagnetic radiation in the infra-red (usually mid- or near-infrared) region corresponding to discrete vibrational transitions of functional groups of the molecule in the ground electronic state. In the case of Raman spectroscopy, the sample is excited by a laser in the ultraviolet, visible, or near-infrared region, bringing the molecule to a higher energy state, prior to its return to a lower energy state by emission of a photon. Raman (inelastic) scattering results when the energy of emission differs from that of the exciting laser, again corresponding to discrete vibrational transition. However, while infra-red absorption and Raman scattering processes both involve vibrational energy levels primarily related to stretching or bending deformations of bonds, infra-red absorption requires a change in intrinsic dipole moment with molecular vibration while Raman scattering depends on changes in polarizability. The two techniques are complementary, with polar functional groups showing stronger signals in infra-red spectra, and non-polar groups being associated with more intense Raman bands. In addition, water has a strong infra-red absorption band which must be subtracted from infra-red spectra of aqueous samples; in contrast, water is a poor Raman scatterer, and therefore Raman spectroscopy is often advantageous for the study of food systems that intrinsically contain high water content [19].

The objective of this paper is to present an overview of vibrational spectroscopy as an analytical tool to investigate structure and molecular interactions of milk proteins, with a focus on the application of Raman spectroscopy to study interactions of proteins and peptides with other protein, lipid or polysaccharide molecules. Recent developments in vibrational spectroscopic techniques indicate promising future applications of this versatile method in dairy science.

2. RAMAN SPECTROSCOPY OF MILK PROTEINS

Table I shows the assignment of characteristic bands in the Raman spectra of proteins that can be used to monitor changes in the secondary structure, microenvironment of aromatic or hydrophobic side chains, reduction of disulfide or oxidation of sulfhydryl groups, or dissociation or ionization of carboxylic acid groups. More comprehensive descriptions of the interpretation of these bands have been reviewed in the literature [19, 20].

Applications of Raman spectroscopic analysis to study milk proteins are

Table I. Assignment of typical bands in Raman spectra of proteins. (Adapted from [19]).

Band assignment	Wavenumber (cm^{-1})
S-S stretch Cystine	510, 525, 545
S-H stretch Cysteine	2550–2580
C-S stretch Cyst(e)ine, Methionine	630–670, 700–745
Tryptophan	760, 880, 1360
Tyrosine doublet	850/830
Phenylalanine	1005
C=O stretch of COO^-, COOH	1400, 1730
C-H bend	1453
-C-H or =C-H stretch	2880, 2930, 3060
α-Helix	938
Amide I	1650–1685
Amide III	1235–1270
O-H stretch	3200

illustrated in the following examples of research from our laboratory.

2.1. Heat-induced fine-stranded and particulate gels

Gels may be formed upon heating solutions of globular whey proteins such as β-lactoglobulin at sufficiently high solute concentration. Depending on parameters such as pH or the nature and species of ions in the protein solutions, the resulting heat-induced gels may be translucent fine-stranded networks or opaque particulate gels, each with distinctive rheological properties [11]. Research is required to study the "food biophysics" of protein gels [8], and Raman spectroscopy can be a useful tool to detect molecular structural changes that may distinguish fine-stranded and particulate gels [11, 12].

Figure 1A shows the Raman spectra of β-lactoglobulin in solution (at pH 7.0, no added salt) and the translucent fine-stranded gel obtained after heating the solution at 80 °C for 60 min, with greater detail in specific regions of the two spectra shown in Figures 1B–E. Translucent gels were also formed by heating β-lactoglobulin under acidic conditions at pH 2.

The decreased intensity ratio of the Tyr doublet at 855/830 cm^{-1} reflects stronger H-bonding in the translucent gel than the solution, while increased intensity of the Trp band at 760 cm^{-1} shows more non-polar or hydrophobic nature of the microenvironment (Fig. 1B). The band near 935 cm^{-1} is assigned to the polypeptide chain in an α-helical conformation; after heating, this band shifted to 944 cm^{-1} reflecting a decrease in helical content in the fine-stranded gel (Fig. 1C). The Amide III (Fig. 1D) and Amide I (Fig. 1E) bands suggest considerable beta-sheet structures were present in the gels.

The Raman spectra of β-lactoglobulin were also examined at pH 5.4, which is close to the isoelectric point of the protein, as well as at pH 7.0 in the presence of 0.1 or 0.3 $mol \cdot L^{-1}$ NaCl (data not shown). Opaque particulate gels were obtained upon heating at 80 °C for 60 min under these conditions. The results showed

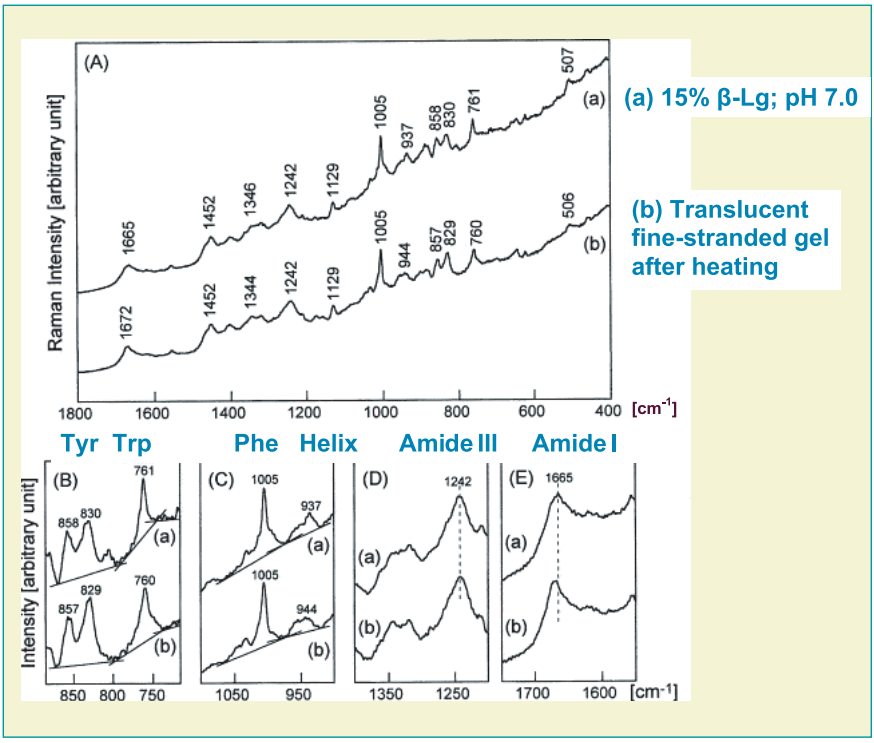

Figure 1. (A) Raman spectra of β-lactoglobulin (15% w/v, pH 7.0) unheated solution (a) and the translucent gel formed after heating at 80 °C for 60 min (b). Lower panel shows the same spectra, but magnified in the regions of (B) Tyr doublet near 850 and 830 cm^{-1} and Trp near 760 cm^{-1}; (C) Phe near 1005 cm^{-1} and α-helix near 937 cm^{-1}; (D) Amide III near 1242 cm^{-1}; and (E) Amide I near 1665 cm^{-1}. (Adapted from [12].)

changes in the Tyr doublet with a much reduced signal at 855 cm^{-1} compared to 831 cm^{-1}, implying strong H-bonding involvement of Tyr residues in these gels. The intensity of the Trp band at 759 cm^{-1} was reduced, indicating exposure to a less non-polar environment, while changes in the Amide I band revealed β-sheet structure in these gels. However, the most dramatic change in the spectrum for the gel compared to the solution was the appearance of a prominent band at 1344 cm^{-1}, which suggested the strong involvement of C-H groups and hydrophobic interactions in the formation of the particulate network of proteins in these gels.

The phenylalanine band near 1003–1005 cm^{-1} has been reported to be insensitive to changes in protein structure and is therefore frequently used as an internal standard for normalization of scattering intensity of other spectral bands. Figure 2 shows the normalized intensities of the Raman spectra of heat-induced gels (in the closed symbols) compared to the unheated solutions (open symbols), at various conditions of pH and ionic strength. Those heated at pH 5.4 and pH 7 with higher salt (0.3 $mol \cdot L^{-1}$) were particulate opaque gels, while the solutions heated at pH 2 or at pH 7 with no added salt yielded translucent fine-stranded gels. The decreases in

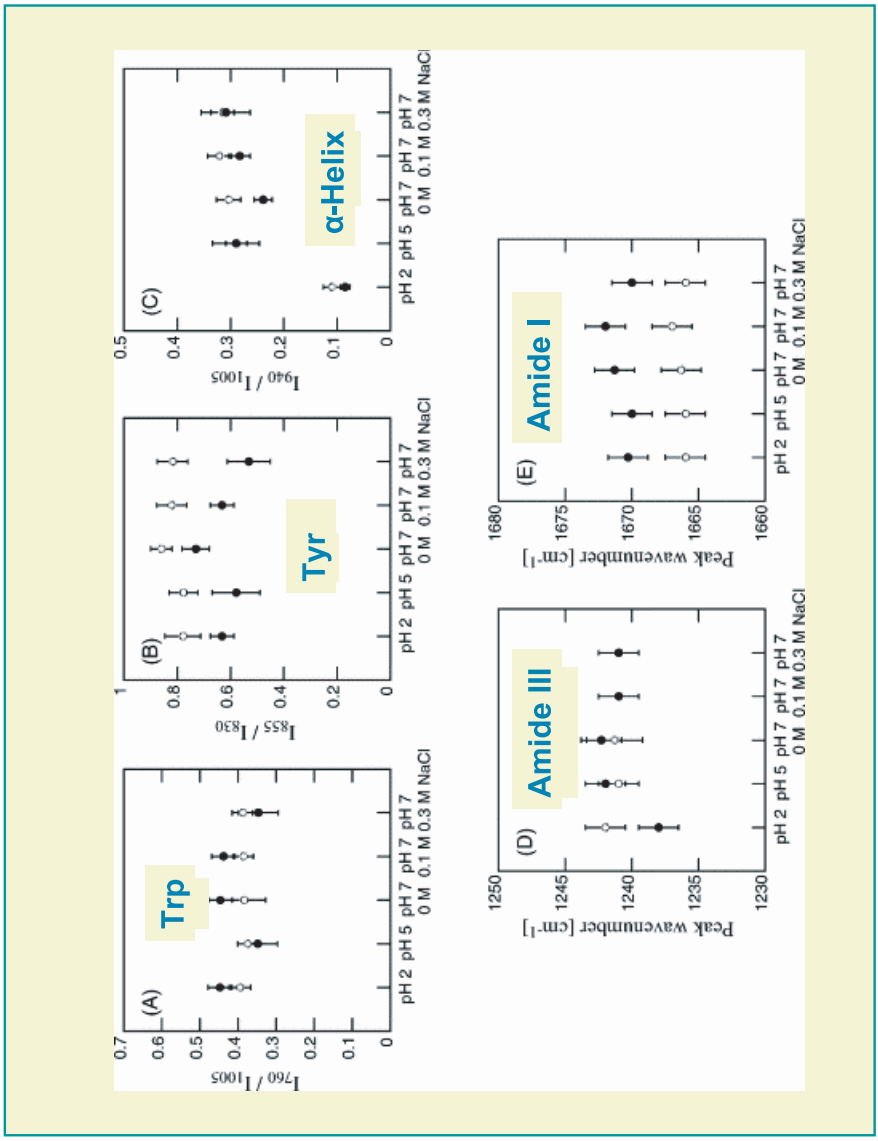

Figure 2. Effects of heat-induced gelation on the normalized intensities of bands assigned to (A) Trp, (B) Tyr doublet ratio, and (C) α-helix, and on the peak wavenumbers assigned to the bands for (D) Amide III and (E) Amide I, for β-lactoglobulin solution (open symbols) and gels (filled symbols) at different conditions of pH and NaCl concentration. (Adapted from [12].)

intensity near 760 cm^{-1} for gels obtained at pH 5.4 and pH 7.0 with no added salt indicate that Trp residues in these opaque gels were in a more exposed or less nonpolar environment, whereas the increased intensity of this band for fine-stranded gels at pH 2.0 or in the presence of salt at pH 7.0 suggests Trp residues in a more hydrophobic environment (Fig. 2A). Decreases in the Tyr doublet ratio intensity after heating in all cases indicate the presence of strong hydrogen bonding of Tyr phenolic groups in the gels, but more so in the particulate gels (Fig. 2B). The lower intensity near 936 cm^{-1} for β-lactoglobulin at pH 2 indicated that the protein had lower helical content even while in solution at this acidic pH, and remained so in the translucent gel; the translucent gel at pH 7 also had lower helical content than the particulate gels (Fig. 2C). The peak locations of the Amide III and Amide I bands in Figures 2D and 2E show that β-lactoglobulin contained β-sheet structure at similar or even higher proportion after heating to form the gels. Interestingly, the peak position of the Amide III band assigned to β-sheet was observed at lower wavenumber for fine stranded gels at pH 2 (Fig. 2D), which may be related to reports of more strongly hydrogen bonded intermolecular sheet structures in these acidic gels [17].

In summary, Raman spectroscopy of the gels formed under different conditions indicated the involvement of aromatic and hydrophobic residues in the gels. Tyr residues were involved in hydrogen bonding in both fine-stranded and particulate gels. Trp residues were in a more hydrophobic microenvironment in fine-stranded gels, in contrast to particulate gels, where they were exposed to a less hydrophobic microenvironment. Hydrophobic interactions affecting C-H bending vibrations were dominant in particulate gels. There was a decrease in helical structure in fine-stranded gels, but considerable retention of beta-sheet structures was evident in both types of gels.

2.2. Protein-lipid interactions at the oil-water interface

The amphiphilic nature of milk proteins enables them to lower the interfacial tension between oil and water, or to form mechanically cohesive interfacial film around oil droplets. For this reason, milk proteins are useful as emulsifiers in foods [9]. However, rather limited information has been published on the structure of proteins at oil-water interfaces, or on the functional groups involved in protein-lipid interactions at the interface. Thirty years ago, Larsson [16] reported on the application of Raman spectroscopy in the C-H stretching region to monitor the fluidity of hydrocarbon chains from milk fat. The hydrocarbon chains of lipids at the oil-water interface or milk fat globule membrane in the milk "emulsion" were concluded to be closely packed and "crystalline" in behavior, in contrast to a more loose packing of the hydrocarbon chains in the isolated milk fat [16].

Recent advances in instrumentation have enabled more detailed studies of protein-lipid interactions at the oil-water interface [23, 24]. The spatial resolution of Raman micro-spectroscopy is determined by the diameter of the beam, and high resolution of 1 μm or better can be achieved using a tightly focused beam. Figure 3 shows the image observed through the microscope of a confocal Raman microscope, with the cross-hair indicating the focus of the exciting laser at the interface between mineral oil (top phase) and 25% bovine serum albumin in phosphate buffer (bottom phase).

Raman spectra were collected at the "zero" point of the interface, as well as at various locations above or below this point. For example, movement of the laser to

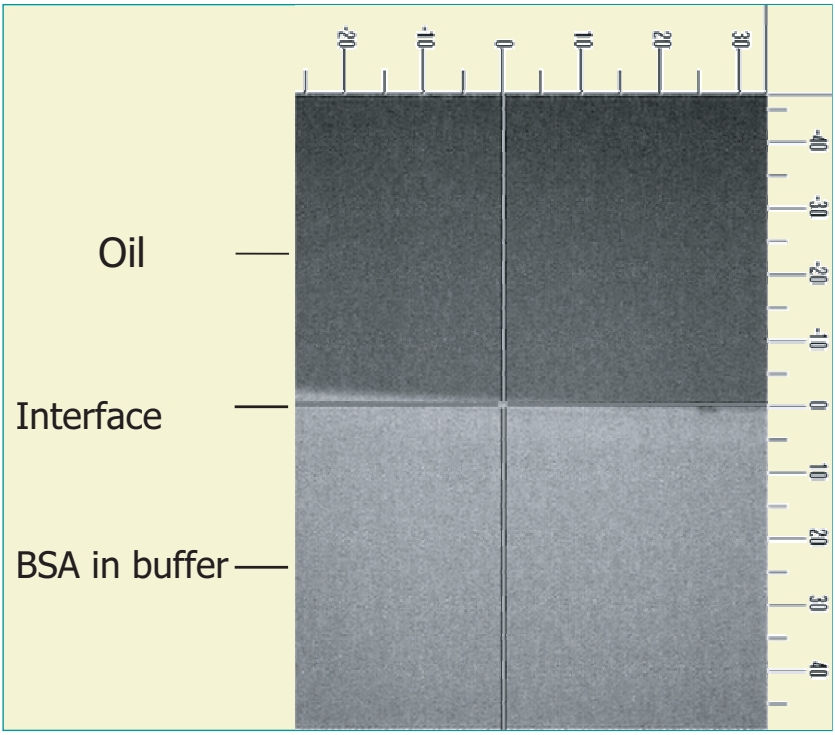

Figure 3. Image of interface between oil and aqueous bovine serum albumin solution viewed using the Raman microscope. Numbers on the scales are in micrometers. (Adapted from [24]).

"–10" enabled collection of Raman scattering at 10 μm toward the oil phase while focusing of the laser at "+10" corresponded to excitation of the sample at a position of 10 μm towards the bovine serum albumin aqueous phase.

Figure 4 shows the spectra for mineral oil, bovine serum albumin solution and at the interface of the two layers; the interface spectrum shows characteristic bands attributed to each of the oil and bovine serum albumin components. One approach to investigate whether interactions occur between the protein and oil components at the interface is by computation of a difference spectrum. For example, Figure 5 shows the results of subtraction of the mineral oil component spectrum from the interface spectrum (Fig. 5b), compared to the bovine serum albumin component spectrum (Fig. 5a). In the absence of interactions between the bovine serum albumin and the oil at the interface, the difference spectrum should be identical to the bovine serum albumin component spectrum. In fact, as shown in Figure 5, interactions between the two components were clearly indicated by changes in various positions in the spectrum, e.g. the Trp band, Tyr doublet ratio, and in the C-H bending/stretching bands.

Raman spectra were similarly obtained for the interface between corn oil and bovine serum albumin solution. The difference spectra indicated only minor changes in the secondary structure of the bovine serum albumin component, but relatively major changes were observed in the

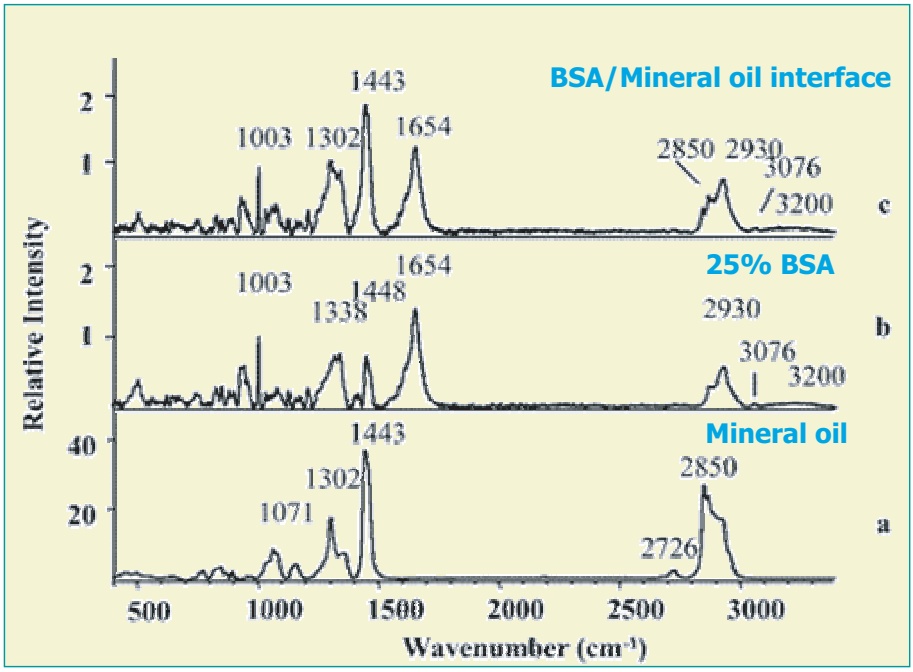

Figure 4. Raman spectra of (a) mineral oil, (b) bovine serum albumin (BSA) in buffer, and (c) the interface between mineral oil and BSA. (Adapted from [24].)

regions associated with C-H bending of both bovine serum albumin and oil, as well as C=C, and COOR stretching modes of the oil [24].

This investigation using Raman microspectroscopy demonstrated that there was little change in the secondary structure of bovine serum albumin at the oil-water interface. Instead, the C-H groups of hydrophobic side chains and aromatic groups of Trp and Tyr residues were involved in the interactions of bovine serum albumin with oil, and more so with mineral oil than with corn oil. Interactions with bovine serum albumin led to changes in the C-C-C backbone stretching vibrations of the hydrocarbon chains of mineral oil at the interface, whereas vibrations assigned to COOR groups of the triacylglycerol, as well as C-H bending and stretching vibrations of CH_2, CH_3 and =CH groups of the fatty acyl chains were implicated in the interactions of corn oil with bovine serum albumin [24].

2.3. Peptide-phospholipid interactions in liposomes

Lactoferricin is a 25-residue cationic antimicrobial peptide derived from the whey protein lactoferrin [9]. Much research has been conducted to understand the mode of action of this peptide against specific pathogens. Ideally, antimicrobial peptides should interact only with membranes of target microbes, and should not disrupt the membrane of mammalian cells as the latter could lead to the undesirable outcome of hemolysis. In the light of the distinctive phospholipid compositions of mammalian cell membranes versus various

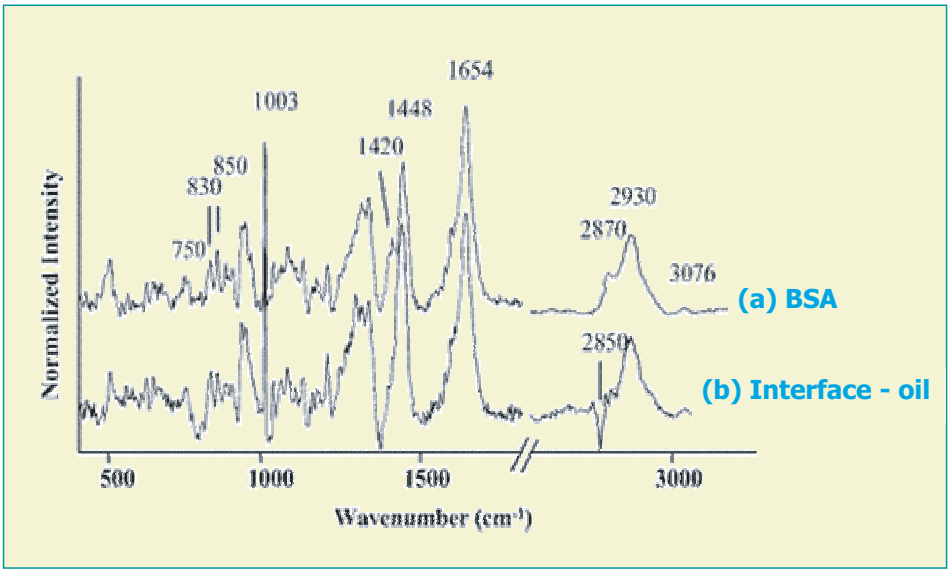

Figure 5. Raman spectrum of (a) bovine serum albumin (BSA) solution, compared to (b) the difference spectrum obtained by subtracting the oil component spectrum from the interface spectrum. (Adapted from [24]).

Gram positive and Gram negative bacteria [31], it is important to investigate the effects of lactoferricin on different phospholipid membrane systems. Raman spectroscopy is a suitable technique that may be used to address this question, since the Raman spectra of phospholipids are sensitive to changes in environmental factors such as temperature or interactions with other substances in the bilayer.

To study the interactions of lactoferricin with phospholipids, Raman spectra of liposomes composed of phospholipids with different head groups and acyl chains were measured in the absence and presence of lactoferricin in the 3000–2850 cm^{-1} C-H stretching region, as a function of varying temperature from –10 to 60 or 70 °C [5].

Figure 6a shows a typical Raman spectrum of phospholipids in the C-H stretching region. The two dominant bands at 2850 and 2880 cm^{-1} arise from the symmetric and asymmetric C-H stretching modes, respectively, of the CH_2 moieties of the hydrocarbon chains of the fatty acyl group, whereas the band near 2935 cm^{-1} is attributed to the symmetric C-H stretching mode of the terminal CH_3 groups of these chains in the hydrophobic centre of the bilayer [4, 18, 35].

The intensity ratios of these three characteristic bands in the C-H stretching region provide information on the fluidity or interaction of the CH_2 groups in the acyl chain as well as the packing order near the terminal CH_3 group. As shown in Figures 6b and 6c, thermally dependent transitions representing the loosening or increased fluidity of the hydrocarbon chains with increasing temperature are reflected by higher 2850/2880 and 2935/2880 intensity ratios, with the former ratio monitoring the order related to lateral chain-chain interactions and the latter ratio related to inter-chain order as well as *gauche* conformations along the lipid chains.

The influence of lactoferricin on model membrane systems was investigated using

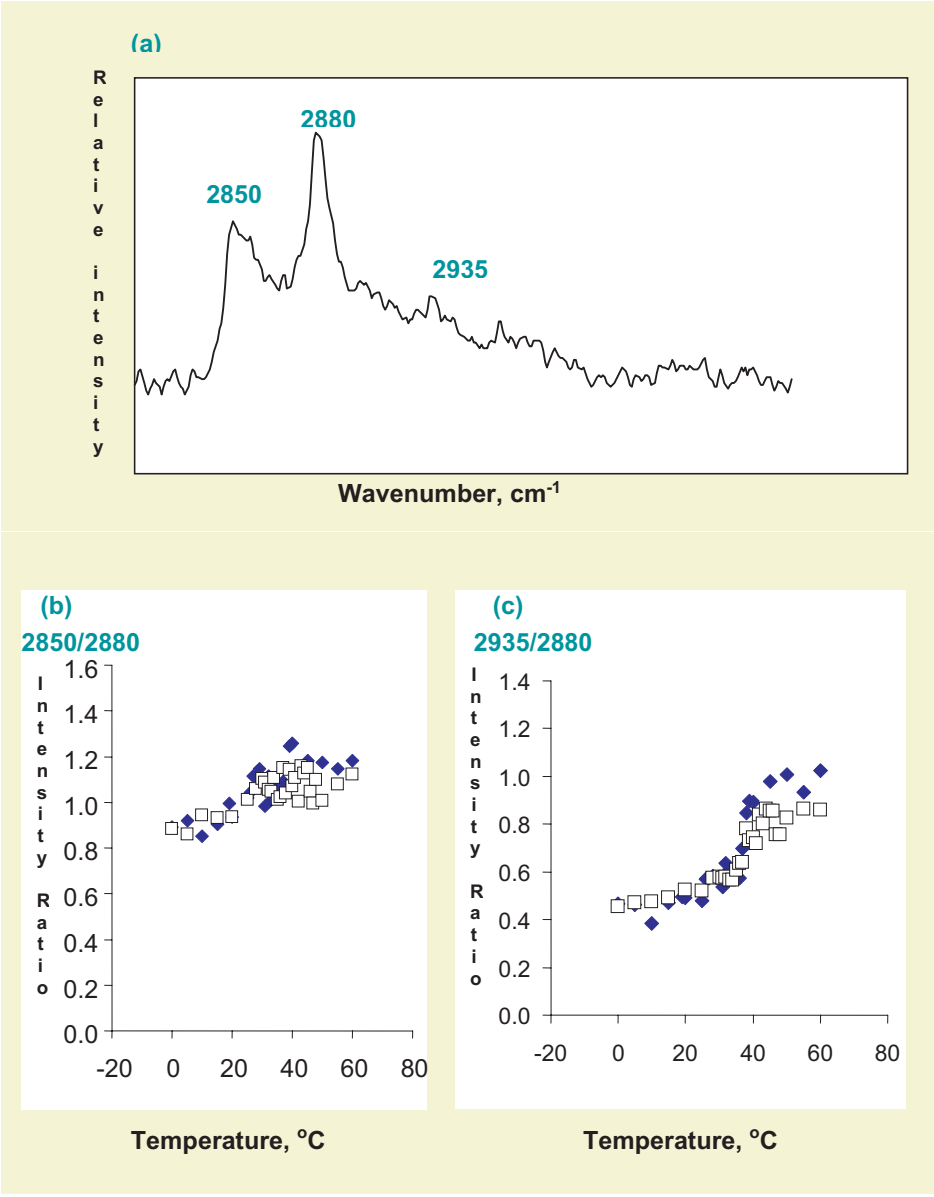

Figure 6. (a) Typical Raman spectrum in the C-H stretching region of phospholipids, and effects of temperature on (b) 2850/2880 and (c) 2935/2880 intensity ratios of DPPG liposomes in the absence (closed symbols) or presence (open symbols) of the antimicrobial peptide lactoferricin. (Adapted from [5].)

Table II. Results of analysis of variance to assess effects of pH, heat treatment, κ-carrageenan and their interactions on characteristic bands in the Raman spectra of bovine serum albumin. (Adapted from [1]).

Raman band assignment	Significant main effect or interaction	Significance level (*P*-value)
α- Helix (938 cm^{-1})	pH	0.015
	Heat	0.017
	Heat × κ-carrageenan	0.035
α- Helix (Amide I band)	Heat	0.011
β-Sheet (Amide I band)	Heat	0.028
C-H stretch (2880 cm^{-1})	Heat	0.010
	Heat × κ-carrageenan	0.035
C-H stretch (2930 cm^{-1})	Heat	0.042

liposomes composed of phospholipids with zwitterionic (phosphatidyl choline PC and phosphatidyl ethanolamine PE) or negatively charged (phosphatidyl glycerol PG) head groups, and only saturated (dipalmitoyl DP) or saturated and unsaturated (palmitoyl and oleoyl PO) fatty acyl chains [5]. The presence of lactoferricin resulted in more restricted mobility of acyl chains and slightly greater thermal stability of DPPC liposomes throughout the temperature range examined, while reduced mobility of acyl chains in DPPG liposomes was only observed at temperatures above the transition temperature (~40 °C). In contrast, mobility of the acyl chains in DPPE was increased in the presence of lactoferricin at temperatures below 32 °C. Although lactoferricin had little effect on POPC and POPE, i.e., phospholipids with unsaturated acyl chains and head groups with no net charge, lactoferricin lowered the lateral chain-chain interaction along the acyl chains of POPG with its negatively charged head groups.

Further research by means of this approach to investigate other peptide sequences and phospholipids extracted from the cellular membrane of target microorganisms would be useful to elucidate the interactions and mechanism of action of cationic antimicrobial peptides with specific microbial cells.

2.4. Raman spectroscopy and chemometrics study of whey protein and κ-carrageenan interactions

The interactions of whey proteins with anionic polysaccharides such as κ-carrageenan may lead to textural changes including increased viscosity, gelation or precipitation, depending on conditions such as pH, ionic strength or temperature [27, 33]. Raman spectroscopy is well-suited to investigate these interactions, being applicable to analyze samples in various states.

Raman spectra were measured for whey protein and κ-carrageenan systems at pH 5, 7 and 9, before and after heating at 80 °C for 30 min [1]. Nine characteristic bands in the Raman spectra were assigned to vibrational modes of specific groups in the protein component as described earlier (Tab. I), with changes in intensity or area of each band related to changes in those groups. In order to integrate the information from these nine spectral bands and three additional parameters related to secondary structure

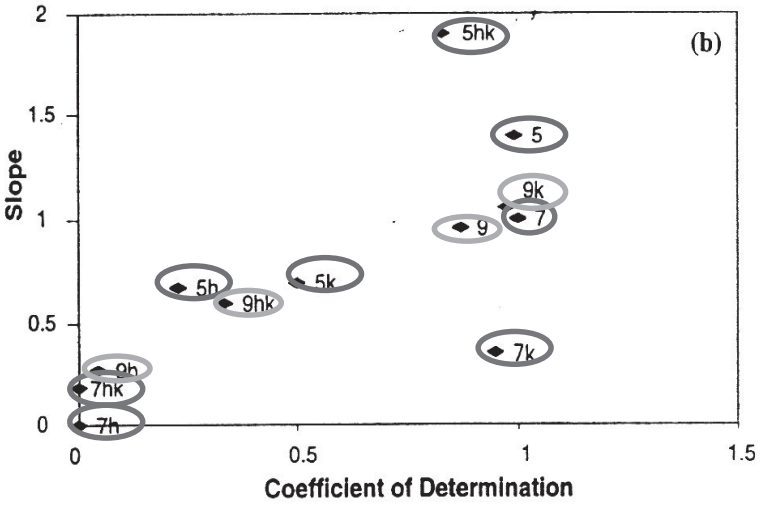

Figure 7. Scattergram obtained by principal component similarity analysis of Raman spectral data for bovine serum albumin at different pH (5, 7 or 9), with or without heat treatment (h) and κ-carrageenan (k). (Adapted from [1].)

estimated from the Amide I band, the Raman spectral data for three whey protein systems (β-lactoglobulin, bovine serum albumin, and whey protein isolate) were analyzed using analysis of variance, principal component analysis and principal component similarity analysis [1]. The results for bovine serum albumin are discussed below to illustrate the analytical approach.

Table II shows that heat treatment significantly ($P < 0.05$) influenced the Raman bands assigned to secondary structure as well as C-H stretching vibrations of bovine serum albumin. The Raman band near 938 cm^{-1} that is characteristic for α-helical structure was also influenced by pH of the system, as well as by the interaction between heat treatment and κ-carrageenan, and the latter interaction term also influenced the symmetric C-H stretching of hydrophobic side chains.

Principal component analysis revealed that 87.2% of the spectral data variability between bovine serum albumin samples under different conditions could be explained by four principal components, with each principal component composed of contributions from several correlated Raman bands. Principal component similarity (PCS) analysis [36] provided a concise way to visualize the treatments on a single plot (Fig. 7). Using this technique, the Raman spectra of bovine serum albumin under different conditions of pH, heating, and/or addition of κ-carrageenan, were evaluated for extent of similarity to the reference spectrum, which in this case was bovine serum albumin at pH 7 in the absence of heat treatment and κ-carrageenan [1]. The Raman spectrum at the reference condition is represented by the point on the PCS scattergram having slope and coefficient of determination values of 1.0. Sample points having coefficient of determination values away from 1.0 in particular would therefore reflect dissimilarity arising from those particular treatments compared to the reference. Thus, for example, at pH 7, heat treatment resulted in much greater change in the Raman spectrum than did the presence of κ-carrageenan, indicating

relatively insignificant interactions of the protein with κ-carrageenan at pH 7. Heating at pH 5 resulted in some changes in the protein, although less so than at pH 7 or 9; however the effect of κ-carrageenan was dominant at pH 5, which may have been due to electrostatic interactions between the anionic polysaccharide molecules with positively charged protein groups at acidic pH. In addition, presence of κ-carrageenan dampened some of the changes induced by heating of bovine serum albumin at pH 5. Adjusting pH to pH 9 without heating caused some changes compared to the reference at pH 7, while heating at pH 9 led to a dramatic change, which was attenuated somewhat by heating in the presence of κ-carrageenan.

These results illustrate the use of chemometrics as an aid in interpreting the important changes in the Raman spectra that are being affected by various treatments or different environmental conditions of the protein.

3. OTHER APPLICATIONS, NEW TECHNIQUES AND TRENDS IN VIBRATIONAL SPECTROSCOPY

The above examples illustrate how vibrational spectroscopy, particularly Raman spectroscopy, may be applied to study the interactions and structural changes of milk proteins or peptides. There are many other applications and trends of vibrational spectroscopy for the study of milk proteins specifically or for more general analysis of milk and milk products e.g. [2, 7, 10, 28, 34]. Fourier transform mid-infrared analyzers are now used widely and routinely in conjunction with data analysis by chemometrics to measure constituents or composition of milk and milk products, including online process testing for rapid process adjustments, such as for standardization of milk fat and protein contents prior to cheese production [2]. Aside from proteins, there also exists a diverse range of applications to other (i.e. non-protein) milk components, such as studies on the crystallinity of lactose in whey powder [29], determination of conjugated linoleic acids in cow's milk fat by FT-Raman spectroscopy [25], or investigation of the effects of sucrose, maltodextrin and skim milk on survival of *L. bulgaricus* after drying [30].

With the advent of new techniques, the potential for future applications is even more promising. Application of near-infrared spectroscopy for protein structure analysis has potential advantages of being suitable for on-line applications for quality control and suffering less serious interference from water [3]. It has been suggested that FT-IR and NIR spectroscopy may complement each other, as FT-IR may be more suitable for monitoring changes in secondary structure, while NIR may be able to detect tertiary and quaternary structure changes [3].

There are many possible applications of infra-red and Raman micro-spectroscopy to study food systems [32]. In situ analysis using fibre optics coupled to infra-red or Raman spectroscopy also has been gaining popularity for minimally invasive medical diagnosis, in vivo biomedical applications [14, 21] and pharmaceutical products [6]. These techniques could show promise for real-time non-destructive monitoring of fluid or solid products during processing, but research on the feasibility and scope of these techniques for milk and milk product analysis is still in its infancy. Moreover, surface-enhanced Raman spectroscopy [26], vibrational Raman optical activity [22], surface plasmon resonance near-infrared spectroscopy [13] or femtosecond stimulated Raman spectroscopy [15] are examples of many recent developments in the paradigm of vibrational spectroscopy which have yet to be explored

for detailed structural investigations of proteins and other constituents in dairy systems.

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