Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 105 (2007) 784–792
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Raman spectroscopic study of amidated food proteins

Hing-Wan Wong [a], David Lee Phillips [b,c], Ching-Yung Ma [a,*]

[a] Food Science Laboratory, Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong
[b] Center for Applied Spectroscopy and Analytical Sciences, The University of Hong Kong, Hong Kong
[c] Department of Chemistry, The University of Hong Kong, Hong Kong

Received 7 September 2006; received in revised form 23 November 2006; accepted 19 January 2007

Abstract

Various amounts of tryptophan were attached to three food protein products: soy protein isolates, spray-dried egg white and gluten, using a water-soluble carbodiimide method. The extent of amidation was determined by a spectrophotometric method. Raman spectra (600–2000 cm^{-1}) of the modified proteins were obtained and analyzed. The phenyl stretching vibration at 1552 cm^{-1}, directly attributed to the attached tryptophan, was used as a marker band, and increases in band intensity were observed in the modified protein samples. Calibration curves were constructed by plotting the intensity ratio of the 1552 cm^{-1} band to the 1003 cm^{-1} phenylalanine stretching band (used as an internal standard) against the amount of tryptophan attached. High correlation coefficients, $(r) \geqslant 0.99$, were obtained from these calibration curves. The Raman spectral data showed a transition from ordered conformation to random coil structures in the amidated food protein products.

Keywords: Amidation; Soy proteins isolates; Spray-dried egg white; Gluten; Raman spectroscopy

1. Introduction

Food proteins can be chemically modified to improve functional properties as well as nutritional value. Proteins from plants and other unconventional sources have limited nutritional value, due to their low content of one or more essential amino acids, and several major studies have been conducted to improve the nutritional value of these proteins. Fortifications of foods and feeds with essential amino acids (Benevenga & Cieslak, 1978), supplementation of cereal proteins by oilseed proteins (Sarwar, Sosulski, Bell, & Bowland, 1978) and enzymatic protein degradation and re-synthesis have been attempted for improving the nutritional quality of proteins (Yamashita, Arai, & Fujimaki, 1976).

Although the fortification of food with free amino acids (Altschul, 1974) is widely used in formulating animal feeds, disadvantages associated with the addition of free amino acids to food proteins have been encountered (Bressani, 1988; Yamashita, Arai, Tsai, & Fujimaki, 1970). Therefore, covalent attachment of limiting essential amino acids to proteins could eliminate these problems and is therefore of great interest to the food industry. The carbodiimide condensation reaction is a useful method for covalently linking essential amino acids, e.g. tryptophan, to food proteins. Voutsinas and Nakai (1979) successfully used 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide to increase tryptophan content of soy protein isolates by 11.3-fold.

The determination of tryptophan content in amidated food proteins is important, since changes in protein conformation and functional properties are influenced by the level of modification. For example, Puigserver, Sen, Gonzales-Flores, Feeney, and Whitaker (1979) reported that tryptophylcasein had altered conformation and decreased solubility. Tryptophan in food proteins can be determined by hydrolysis, followed by amino acid analysis. Hydrolysis can be achieved by using hydrochloric acid with additives

* Corresponding author. Tel.: +852 2299 0318; fax: +852 2858 3477.
E-mail address: macy@hkucc.hku.hk (C.-Y. Ma).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.01.040

(Andersen, Mason, & Bech-Andersen, 1984), organic acids (Liu & Chang, 1971), or bases (Allred & MacDonald, 1988). Relatively direct methods are also available, including the acid ninhydrin method (Pintér-Szakács & Molnár-Perl, 1990) and *N*-bromosuccinimide method (Spande & Witkop, 1967). However, samples are destroyed in all these procedures. Hence, the development of a simple, rapid and non-destructive analytical method for determining the extent of amidation in protein samples is highly desirable. Raman spectroscopy has recently been used in our laboratory to determine the degree of acetylation and succinylation in three food proteins (Zhao, Ma, Yuen, & Phillips, 2004a, 2004b). The advantages of Raman spectroscopy in studying food proteins are that samples can be analyzed both in liquid and solid states and, in many cases, without destroying the samples. In addition, Raman spectroscopy can provide qualitative information on food proteins. The attachment of amino acids to proteins will disturb the electrostatic states and alter the conformation.

Since the functional properties of food proteins are closely related to their physicochemical and structural properties, obtaining conformational information on amidated proteins is important since the modified proteins are used, not only for improving nutritional value, but also for enhancing functionality.

In this investigation, a rapid and non-destructive Raman spectroscopic method is developed, to determine the extent of modification in tryptophan-amidated protein products: soy protein isolates, spray-dried egg white and gluten. Conformational changes under the influence of amidation are also studied by examining the Raman spectral data.

2. Materials and methods

2.1. Materials

Commercial soy protein isolates (SPI), Supro 610, were obtained from Protein Technology International (St. Louis, MO, USA). Spray dried egg white (EW) powders were obtained from the Canadian Inovatech Inc. (Abbotsford, BC, Canada). Gluten (crude, from wheat, approximately 80% protein) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used in this study were of analytical grade.

2.2. Preparation of amidated proteins

Protein samples were amidated according to the method of Murphy and Howell (1991) and Voutsinas and Nakai (1979) with some modifications. Protein samples (2 g) were mixed with L-tryptophan (1 g) in distilled water (100 ml) and stirred at room temperature for 15 min. Two ml of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) (0.5% w/v in distilled water) were then added to the protein solution. The pH was maintained at 4–5 by adding 1 N HCl. The reaction was stopped by the addition of glacial acetic acid to 1 N concentration. Subsequently, the sample was dialyzed at 4 °C against 1 N acetic acid and then distilled water. After dialysis, the modified protein solution was treated with hydroxylamine (0.5 M) at pH 7.0. The reaction mixture was allowed to stand for 5 h at 25 °C and dialyzed once more against distilled water. Then, the sample was freeze-dried. A control was prepared without adding L-tryptophan.

2.3. Determination of extent of amidation by wet chemistry method

The tryptophan contents of the three protein samples were determined by the spectrophotometric method of Spande and Witkop (1967) with some modifications. The analysis was carried out by placing about 2 ml of a centrifuged, buffered protein solution in a quartz cuvette with a stirring bar. As the bar does not obstruct the light path, it can be retained in the cell during the entire period. Sufficient protein was required to yield an initial absorbance at 280 nm of 1.5–2.0 (1–2 mg/ml was usually required) when measured against the buffer blank.

After recording the initial absorbance at 280 nm, the cuvette was removed from the spectrophotometer (Spectronic Genesys 2) (Milton Roy Company, USA), and magnetic stirring was initiated after adding 5 or 10 μl of a 5–10 mM aqueous solution of *N*-bromosuccinimide (NBS) to the mixture. After stirring for several minutes, the cuvette was placed back in the spectrophotometer and the absorbance at 280 nm was again measured. The addition of NBS was continued in this stepwise fashion until no further decrease in absorbance was recorded, and a slight increase in absorbance may reflect the onset of bromination of the aromatic ring of tyrosine, with dienone formation. Both the reagent and the product, succinimide, displayed negligible absorption at 280 nm. Minimum absorbance recorded was corrected for volume increase due to the addition of reagent (usually 100–200 μl).

As the molecular weight of the protein was not known, the tryptophan content was expressed as a percentage of the weight of the protein as

$$\%\ \mathrm{Trp} = \{(\Delta A_{280} \times 1.31 \times V \times 186)/W \times 5500\} \times 100$$

where ΔA_{280} = corrected absorbance decrease at 280 nm, V = initial volume of titrated solution (ml), W = weight of protein titrated (mg), and % Trp = g tryptophan residues/100 g protein.

2.4. Raman spectroscopy

Raman spectra were recorded on a Renishaw-Raman Imaging Microscope (system 1000) equipped with a 514 nm Argon ion laser excitation (Spectra Physics, Mountain View, CA, USA). Raman frequency was calibrated by a silicon slide at 520 cm^{-1}. The laser was focussed on the solid samples which were dispersed on microscope slides.

Each spectrum was collected at room temperature under the following conditions: 100 mW of laser power, 2 cm^{-1} resolution, 30 s exposure time, and 10 scans. The experiments were performed in a dark environment to avoid interference from light and cosmic radiation.

Spectral data from the scans of samples were baseline-corrected and normalized against the phenylalanine band at 1003 ± 1 cm^{-1} using the GRAMS/32 AI Software (Galactic Industries Corporation, Salem, NH, USA). Raman spectra (400–2000 cm^{-1}) were plotted as relative intensity (arbitrary units) against Raman shift in wavenumber (cm^{-1}). Raman spectra of each sample were collected in duplicate or triplicate and the results were recorded as the averages of these replicates.

Raman calibration curves were obtained by plotting the ratio of the maker band at 1780 cm^{-1} to the 1003 cm^{-1} phenylalanine band (used as an internal standard) against the extent of amidation determined by the spectrophotometric method.

3. Results and discussion

3.1. Determination of extent of amidation by wet chemistry method

Table 1 shows the extent of amidation of SPI, EW and gluten at different reaction time intervals. Although carbodiimides show a preference for carboxyl groups in acid or neutral aqueous solutions at room temperature (Franzblau, Gallop, & Seifter, 1963; Riehm & Scheraga, 1966), complicating side reactions have been reported with tyrosine groups (Carraway & Triplett, 1970; Perfetti, Anderson, & Hall, 1976), sulfhydryl groups (Carraway & Triplett, 1968) and serine groups (Banks, Blossey, & Shafer, 1969). To avoid unwanted reactions, two precautions were taken. First, hydroxylamine was added to prevent reactions between EDC with tyrosine and serine residues (Banks et al., 1969; Carraway & Triplett, 1970). Second, excess amounts of tryptophan and protein samples with little water-soluble carbodiimide were used to avoid *O*-acylisourea and *N*-acylurea formation (Perfetti et al., 1976). However, reaction between EDC and sulfhydryl groups may still occur.

Table 1
Extent of modification and Raman intensity ratio in amidated soy protein isolates (SPI), egg white (EW) and gluten

Protein samples	Reaction time (h)	Extent of amidation (% Try = g tryptophan/100 g protein)	I_{1552}/I_{1003}
SPI			
Control (A)	0	1.3 ± 0.2[a]	0.426 ± 0.002[a]
Sample 1 (B)	0.5	3.1 ± 0.1	0.522 ± 0.003
Sample 2 (C)	1	3.4 ± 0.2	0.556 ± 0.002
Sample 3 (D)	3	4.8 ± 0.15	0.607 ± 0.002
Sample 4 (E)	6	6.2 ± 0.12	0.681 ± 0.004
EW			
Control (A)	0	1.82 ± 0.15	0.472 ± 0.002
Sample 1 (B)	0.5	2.3 ± 0.1	0.498 ± 0.003
Sample 2 (C)	1	3.4 ± 0.1	0.535 ± 0.004
Sample 3 (D)	3	4.4 ± 0.2	0.564 ± 0.003
Sample 4 (E)	6	7.5 ± 0.1	0.678 ± 0.002
Gluten			
Control (A)	0	1.2 ± 0.1	0.293 ± 0.002
Sample 1 (B)	1	1.4 ± 0.15	0.320 ± 0.003
Sample 2 (C)	3	1.7 ± 0.1	0.345 ± 0.004
Sample 3 (D)	8	2.1 ± 0.2	0.372 ± 0.003
Sample 4 (E)	11	2.6 ± 0.15	0.412 ± 0.002

[a] Means of triplicate determinations ± standard deviations.

3.2. Determination of extent of amidation by Raman spectroscopy

Fig. 1 shows a typical Raman spectrum of native gluten with some major Raman bands assigned. Tryptophan exhibits Raman bands at 577, 761, 879, 1014, 1338, 1363, 1552 and 1582 cm^{-1} (Tu, 1986). The bands at 757, 1363 and 1552 cm^{-1} showed marked increases after modification (Figs. 2–4). The signals at 757 and 1363 cm^{-1} were not as good as that at 1552 cm^{-1} for use as marker bands to compare with the spectrophotometric data. This may be due to the fact that the 1552 cm^{-1} band is attributed to tryptophan only, while the other transitions are attributed to mixtures of tryptophan and C–H bending vibrations (Tu, 1986).

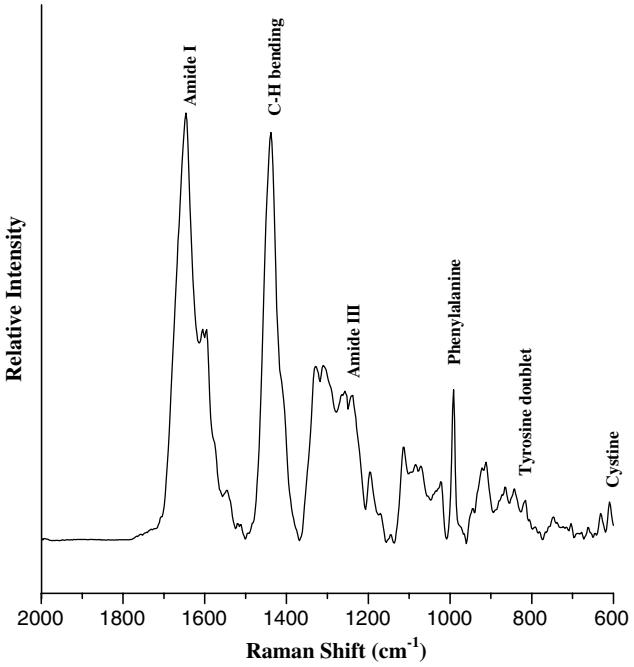

Fig. 1. Raman spectrum of unmodified gluten.

As mentioned previously, EDC can also react with cysteine, serine and tyrosine, forming undesirable side products. Although hydroxylamine was added to regenerate serine and tyrosine, cysteine side reactions may still occur. However, both the wet chemistry and Raman spectroscopic methods measure the signal directly from tryptophan in the samples, and the side reactions between EDC and other amino acids will not affect the measurements. Since the 1552 cm^{-1} band can be directly compared with spectropho-

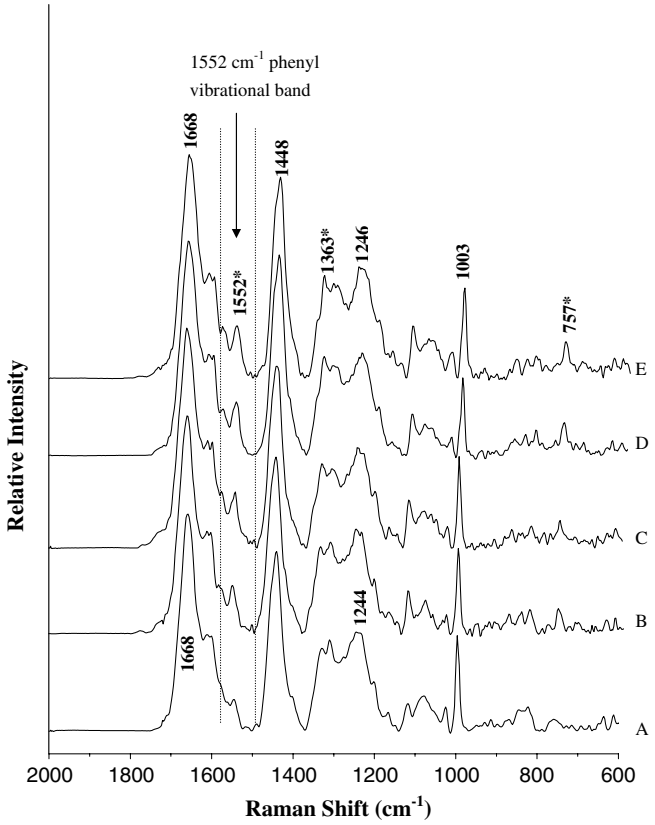

Fig. 2. Raman spectra of amidated soy protein isolates: (A) unmodified; (B–E) amidated to increasing extent (see Table 1).

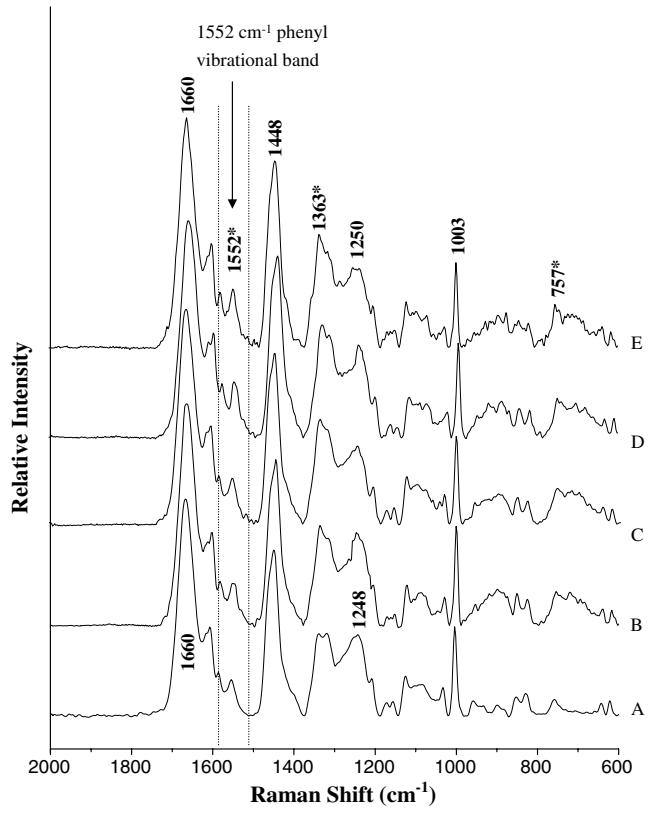

Fig. 3. Raman spectra of amidated egg white: (A) unmodified; (B–E) amidated to increasing extent (see Table 1).

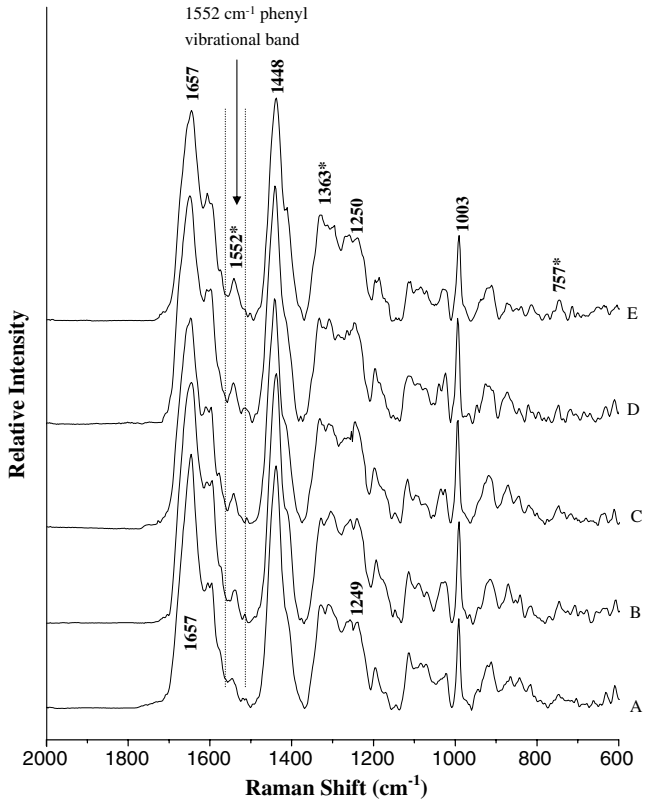

Fig. 4. Raman spectra of amidated gluten: (A) unmodified; (B–E) amidated to increasing extent (see Table 1).

tometric data without interference from side reactions, it was therefore selected as a marker band for the construction of calibration curves. Although this band was not completely resolved from the neighboring peak, the use of peak height for plotting calibration curves was found to be satisfactory. Spectral data processing, such as deconvolution, has been attempted, but the resulting correlations were not as good as those using peak heights alone.

Table 1 lists the ratio of the intensity of the Raman 1552 cm^{-1} vibrational band to the 1003 cm^{-1} phenylalanine band (used as an internal standard) for the three protein samples. Results indicate progressive increases in intensity of the 1552 cm^{-1} band with increasing extent of amidation. Plots of the Raman band ratios against extent of amidation are shown in Fig. 5.

Table 2 shows the calibration equations ($y = mx + b$; where y is the ratio of 1552 cm^{-1} band to 1003 cm^{-1} band, x is the extent of amidation, m is the slope and b is the y-intercept) for each protein. The calibration curves have slopes of 0.0513, 0.0354 and 0.0817 for SPI, EW and gluten, respectively. The y-intercept varied in value from 0.201 to 0.412 with correlation coefficients (r) $\geqslant$ 0.99 (Table 2). The high r values indicate a very strong linear correlation between the I_{1552}/I_{1003} intensity and the extent of amidation for each of the three proteins. It should be noted that the marker Raman band has a background from the native proteins. This background was mainly attributed to the phenyl groups of tryptophan of the parent proteins.

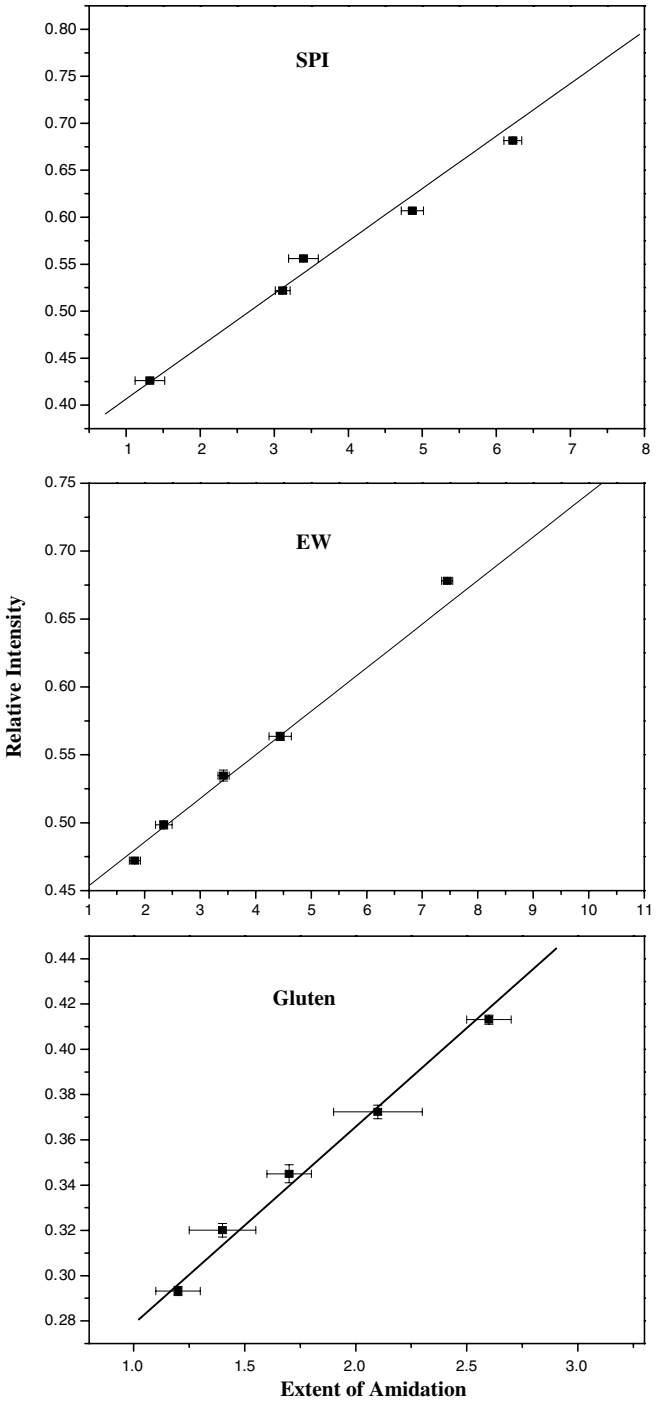

Fig. 5. Plot of the ratio of Raman 1552 cm^{-1} band to 1003 cm^{-1} phenylalanine band against the extent of amidation for soy protein isolates (SPI), egg white protein (EW), and gluten. Error bars represent standard deviations of the Raman (*y*-axis) and wet chemistry (*x*-axis) measurements.

Table 2
Linear regression parameters from the calibration curves of soy protein isolates, egg white and gluten (Fig. 5)[a]

Protein samples	*m*	*b*	*r*
Soy protein isolates	0.0513	0.366r	0.991
Egg white	0.0354	0.412	0.998
Gluten	0.0817	0.201	0.990

[a] $y = mx + b$; where y is the ratio of the 1552 cm^{-1} band to 1003 cm^{-1} band, x is the extent of amidation, m is the slope, and b is the y-intercept. Separate sets of data were fitted to different regression equations for individual proteins.

3.3. Effect of amidation on protein conformation

3.3.1. Backbone conformation

A Raman spectrum can provide valuable information on protein conformation under the influence of chemical modification, as the amide bond has several distinct vibrational modes, i.e. amide I and amide III, in the spectrum, which are commonly used to characterize backbone conformation, giving information on the relative proportions of different types of secondary structures in proteins (Li-Chan, 1996). Generally, proteins with a high proportion of β-sheet structures show an amide I band at 1667–1673 cm^{-1} and a more intense amide III band near 1230–1240 cm^{-1}. Proteins with predominantly high α-helical contents show an amide I band centred at around 1650–1660 cm^{-1}, and a weak amide III band located in a broad region from 1260 to 1300 cm^{-1} region. Proteins containing high contents of undefined or random coil structures have an amide I band close to 1665 cm^{-1} and an amide III transition near 1245 cm^{-1}. The Raman amide I and amide III bands in native soy protein isolates and egg white have been described previously (Zhao et al., 2004a).

In the unmodified gluten (Fig. 4), amide I and amide III bands were centred at 1657 cm^{-1} and 1249 cm^{-1}, respectively, indicating that α-helices are the major secondary structures. This is in agreement with Lee, Haris, Chapman, and Mitchell (1990) who showed that the secondary structure composition of native gluten was 28% β-sheet, 27% β-turns, 17% random coils, and 31% α-helices.

Generally, WPI and EW (animal proteins) have higher tryptophan contents than have SPI and gluten (plant proteins). Hence, native WPI exhibited very strong Raman signals at 1552 and 757 cm^{-1} (Zhao et al., 2004a). Gluten has less tryptophan and exhibited lower intensity for the two Raman bands.

Attachment of tryptophan to food proteins may affect electrostatic interactions and cause conformational changes. Figs. 6–8 show increased intensities in the amide I and amide III transitions in the three amidated protein products, suggesting conformational changes. Moreover, a progressive shift to higher wave number in the amide III band was observed in the spectra of all three proteins (Figs. 2–4), suggesting a transformation to random coil structures. Similar results have been reported for amidated bovine β-lactoglobulin (Mattarella, Creamer, & Richardson, 1983).

Although attachment of tryptophan to proteins by amidation may lead to alteration in conformation, which could potentially affect the Raman signal intensity of tryptophan, we observed that tryptophan band intensity, especially the 1552 cm^{-1} band, in the three protein products, was relatively insensitive to conformational changes. When these

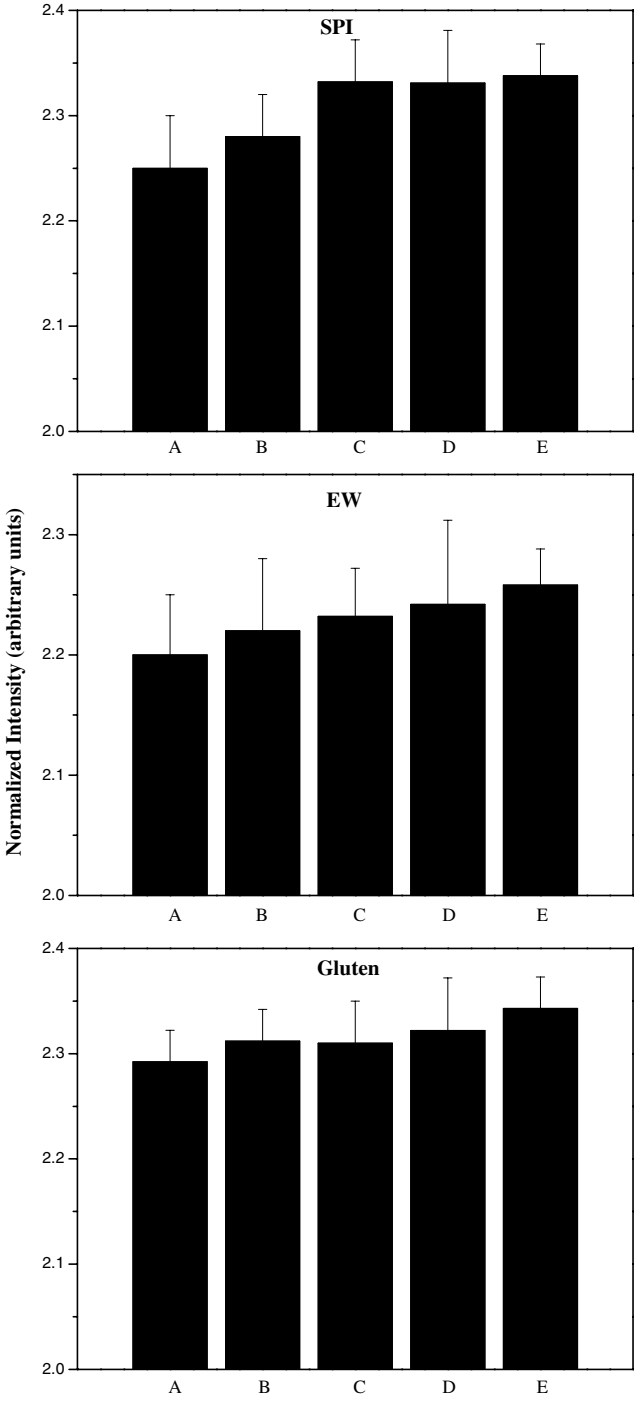

Fig. 6. Effect of amidation on the normalized intensity of Raman amide I band in soy protein isolates (SPI), egg white (EW) and gluten: (A) unmodified proteins; (B–E) proteins amidated to increasing extent (see Table 1). Error bars represent standard deviations of triplicate measurements.

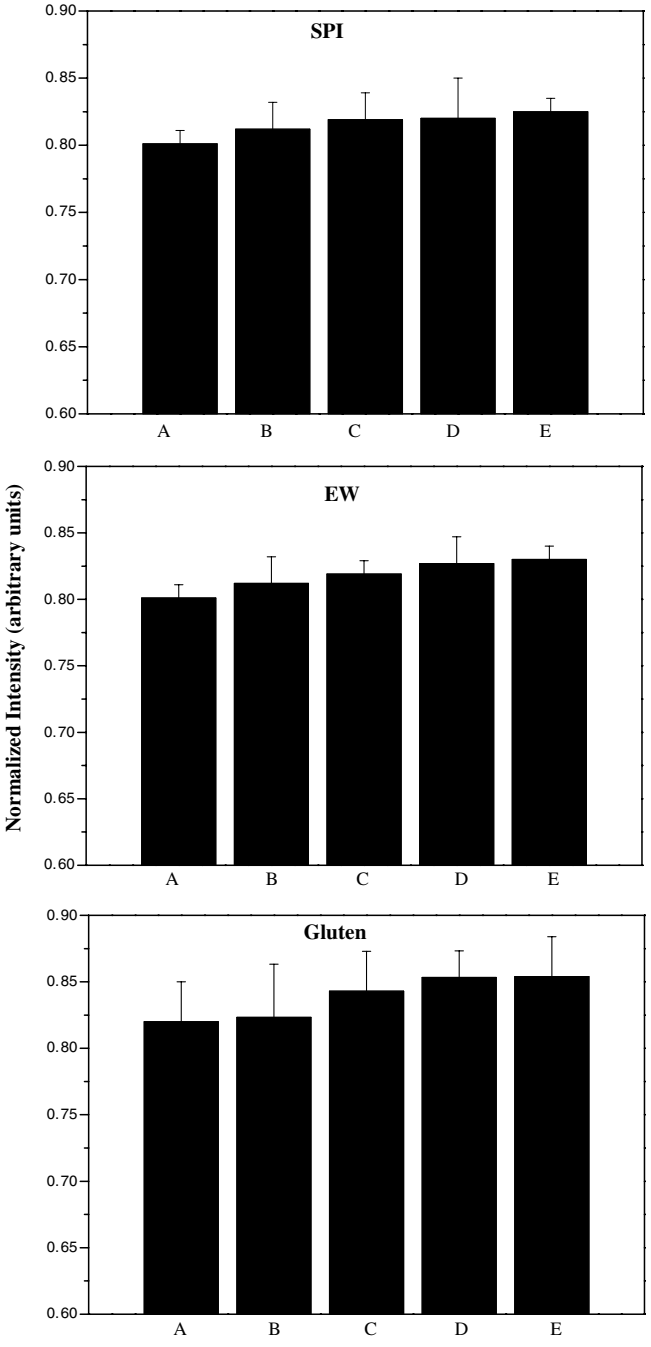

Fig. 7. Effect of amidation on the normalized intensity of Raman amide III bands in soy protein isolates (SPI), egg white (EW) and gluten: (A) unmodified proteins; (B–E) proteins amidated to increasing extent (see Table 1). Error bars represent standard deviations of triplicate measurements.

proteins were modified by deamidation, sulfitolysis, and trypsin hydrolysis, no changes in the tryptophan band intensity were observed except at the highest level of modification, when a slight decrease in peak intensity was recorded (Wong, 2006). On the other hand, marked changes in Raman spectral characteristics were demonstrated in these proteins, even at lower levels of modification. The results suggest that the progressive increases in the 1552 cm^{-1} band intensity in the present study were mainly due to attachment of tryptophan residues to the proteins, rather than changes in conformation or the polypeptide backbone.

3.3.2. Side chain vibrations

Spectra of amidated protein products show a progressive increase in C–H bending intensity (Fig. 8) suggesting

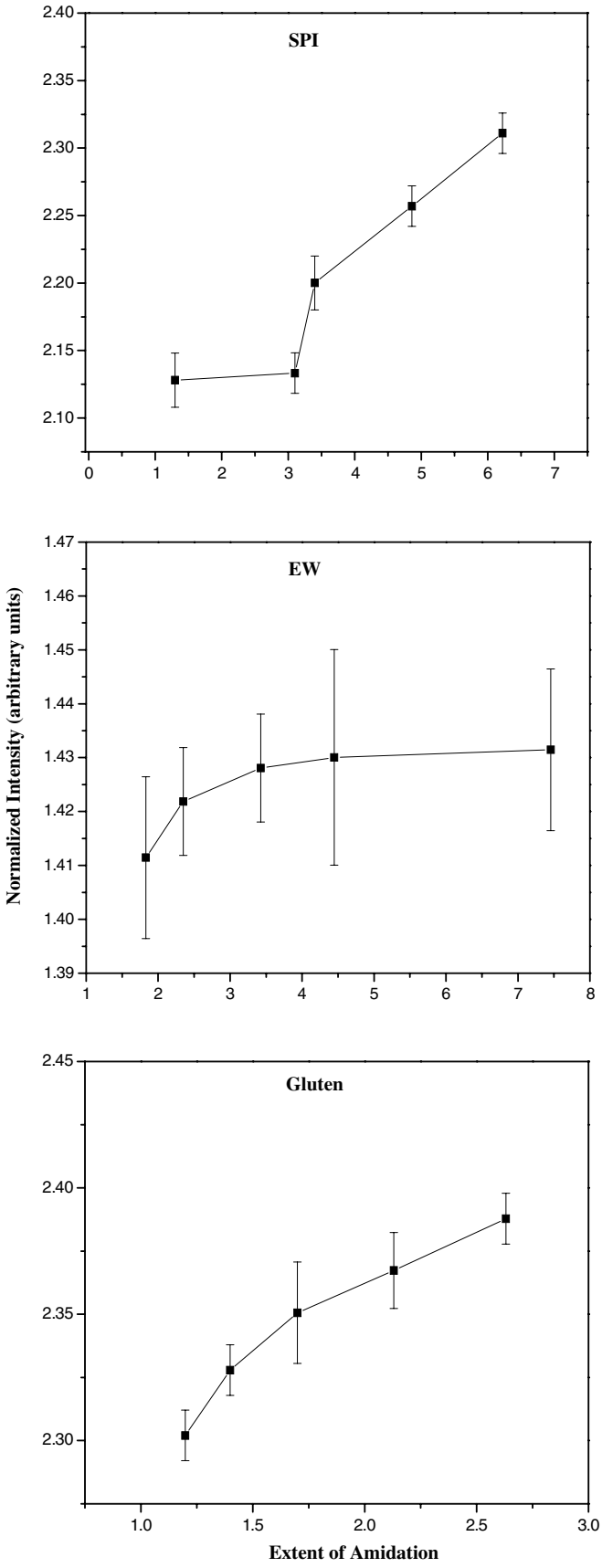

Fig. 8. Effect of amidation on the normalized intensity of Raman 1448 cm^{-1} band in soy protein isolates (SPI), egg white (EW) and gluten. Error bars represent standard deviations of triplicate measurements.

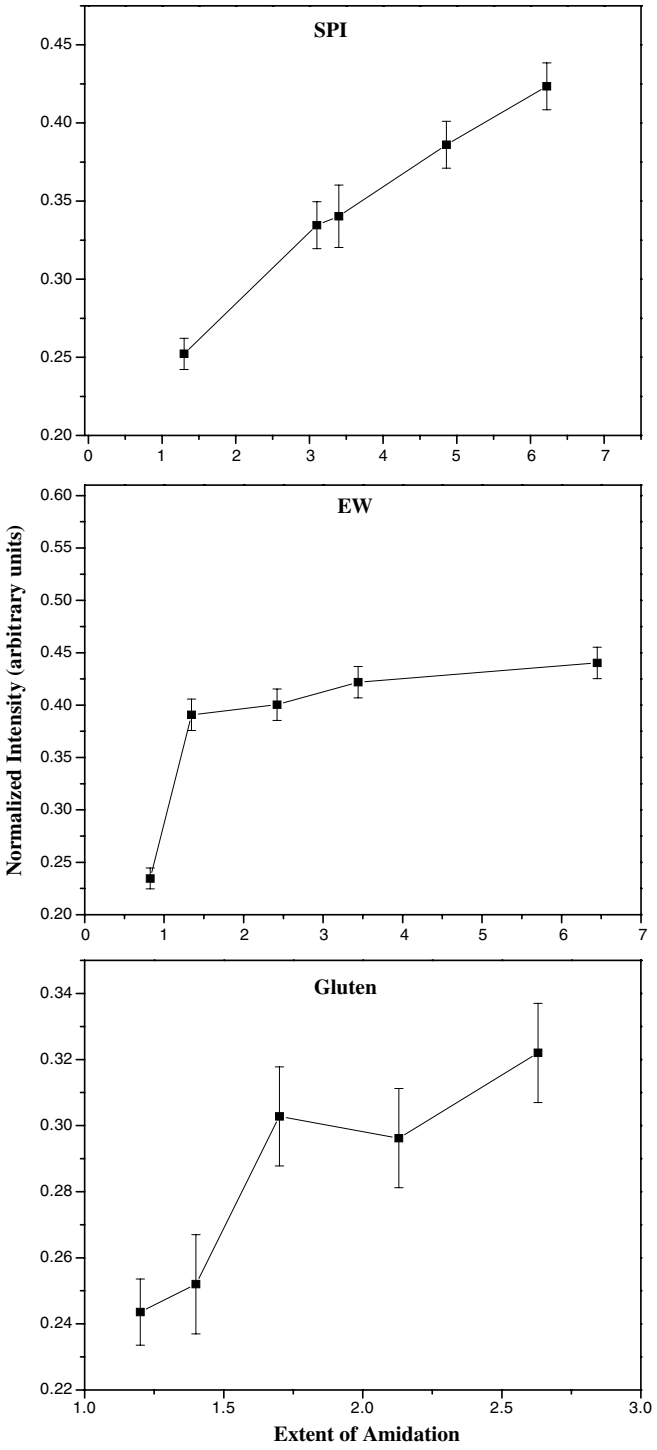

Fig. 9. Effect of amidation on normalized intensity of Raman 757 cm^{-1} band in soy protein isolates (SPI), egg white (EW) and gluten. Error bars represent standard deviations of triplicate measurements.

exposure of hydrophobic groups to a more polar environment (Lippert, Tyminski, & Desmeules, 1976). Attachment of bulky non-polar tryptophan residues to proteins may disrupt electrostatic interactions, leading to partial unfolding of the molecules and exposure of surface hydrophobic groups. It should be noted that the increases in intensities of amide I and amide III bands in amidated SPI and EW

were less than those observed in deamidated samples (Wong, 2006), suggesting different effects of the two modifications on protein conformation.

As discussed previously, attachment of tryptophan to proteins could enhance the original Raman signals. Therefore, progressive increases in intensities of the 1552, 880 and 757 cm^{-1} bands were observed in all three protein products (Figs. 2–4 and 9). The tyrosine I_{850}/I_{830} doublet band intensity cannot be clearly identified in the Raman spectrum of gluten and was not analyzed. For SPI, Raman bands between 800 and 980 cm^{-1} were masked by noise, possibly due to signals from the added tryptophan. Although EW showed a notable signal of tyrosine doublet band, noise from added tryptophan interfered with the measurements. Therefore, the tyrosine doublet band intensity was not analyzed in any of the three amidated samples. The disulphide region was also not analyzed since Raman bands below 600 cm^{-1} were not clearly identified in the spectra.

4. Conclusions

In the present investigation, we demonstrated the potential applications of Raman spectroscopy in studying effects of amidation, a common method for improving nutritional quality of proteins, on three widely used food protein products. Several signals attributed to tryptophan appeared and the 1552 cm^{-1} band was selected for the construction of calibration curves. Careful selection of characteristic Raman bands of a particular modification is important for construction of calibration curves with good correlation coefficients. The marker band selected should preferably be relatively insensitive to changes in conformation, and precautions should be taken to ensure that intensity changes in the marker band were due to chemical derivatization, rather than conformational changes. This can be confirmed by examining Raman spectra of proteins extensively denatured by other means, such as thermal treatments or addition of protein structure perturbants (e.g., urea). The Raman spectral data show that amidation led to increased disordered structures in the three proteins, possibly due to the attachment of the bulky non-polar tryptophan residue.

Acknowledgements

Financial supports were received from a Hong Kong University Conference and Research Grants Committee grant to C.Y.M., Hong Kong Research Grant Council awards to C.Y.M. and D.L.P., and a Faculty Collaborative Research grant to C.Y.M. and D.L.P.

References

Allred, M. C., & MacDonald, J. L. (1988). Determination of sulfur amino acids and tryptophan in foods and food and feed ingredients: Collaborative Study. *Journal of the Association of Official Analytical Chemists, 71*(3), 603–606.

Altschul, A. M. (1974). Fortification of foods with amino acids. *Nature, 248*(5450), 643–646.

Andersen, S., Mason, V. C., & Bech-Andersen, S. (1984). EEC Collaborative studies on a streamlined hydrolysate preparation method for amino acid determinations in feedstuffs. *Zeitschrift Fur Tierphysiologie Tierernahrung Und Futtermittelkunde, 51*(3), 113–129.

Banks, T. E., Blossey, B. K., & Shafer, J. A. (1969). Inactivation of a-chymotrypsin by a water-soluble carbodiimide. *Journal of Biological Chemistry, 244*(23), 6323–6333.

Benevenga, N. J., & Cieslak, D. G. (1978). Some thoughts on amino acid supplementation of proteins in relation to improvement of protein nutriture. *Advances in Experimental Medicine and Biology, 105*, 379–400.

Bressani, R. (1988). Protein complementation of foods. In R. S. Harris & E. Karma (Eds.), *Nutritional evaluation of food processing* (pp. 627–653). Westport: Avi Publishing Co.

Carraway, K. L., & Triplett, R. B. (1968). Reaction of tyrosine residues in proteins with carbodiimide reagents. *Biochimica et Biophysica Acta, 160*(2), 272–274.

Carraway, K. L., & Triplett, R. B. (1970). Reaction of carbodiimides with protein sulfhydryl groups. *Biochimica et Biophysica Acta, 200*(3), 564–566.

Franzblau, C., Gallop, P. M., & Seifter, S. (1963). The presence in collagen of γ-glutamyl peptide linkages. *Biopolymers, 1*(1), 79–97.

Lee, D. C., Haris, P. I., Chapman, D., & Mitchell, R. C. (1990). Determination of protein secondary structure using factor analysis of infrared spectra. *Biochemistry, 29*(39), 9185–9193.

Li-Chan, E. C. Y. (1996). The applications of Raman spectroscopy in food science. *Trends in Food Science and Technology, 7*(11), 361–370.

Lippert, J. L., Tyminski, D., & Desmeules, P. J. (1976). Determination of the secondary structure of proteins by laser Raman spectroscopy. *Journal of the American Chemical Society, 98*(22), 7075–7080.

Liu, T.-Y., & Chang, Y. H. (1971). Hydrolysis of proteins with *p*-toluenesulfonic acid. determination of tryptophan. *Journal of Biological Chemistry, 246*(9), 2842–2848.

Mattarella, N. L., Creamer, L. K., & Richardson, T. (1983). Amidation or esterification of bovine b-lactoglobulin to form positively charged proteins. *Journal of Agricultural and Food Chemistry, 31*(5), 968–972.

Murphy, M. C., & Howell, N. K. (1991). Effects of the attachment of small molecules by carbodiimide-mediated condensation on the physicochemical and functional properties of bovine serum albumin. *Journal of the Science of Food and Agriculture, 55*(1), 117–140.

Perfetti, R. B., Anderson, C. D., & Hall, P. L. (1976). The chemical modification of papain with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. *Biochemistry, 15*(18), 1735–1743.

Pintér-Szakács, M., & Molnár-Perl, I. (1990). Determination of tryptophan in unhydrolyzed food and feedstuffs by the acid ninhydrin method. *Journal of Agricultural and Food Chemistry, 38*(3), 720–726.

Puigserver, A. J., Sen, L. C., Gonzales-Flores, E., Feeney, R. E., & Whitaker, J. R. (1979). Covalent attachment of amino acids to casein. 1. Chemical modification and rates of in vitro enzymatic hydrolysis of derivatives. *Journal of Agricultural and Food Chemistry, 27*(5), 1098–1104.

Riehm, J. P., & Scheraga, H. A. (1966). Structural studies of ribonuclease: XXI. The reaction between ribonuclease and a water-soluble carbodiimide. *Biochemistry, 5*(1), 99–115.

Sarwar, G., Sosulski, F. W., Bell, J. M., & Bowland, J. P. (1978). Nutritional evaluation of oilseeds and legumes as protein supplements to cereals. *Advance in Experimental Medicine Biology, 105*, 415–441.

Spande, T. F., & Witkop, B. (1967). Determination of the tryptophan content of protein with *N*-bromosuccinimide. *Methods in Enzymology, 11*, 498–506.

Tu, A. T. (1986). Peptide backbone conformation and microenvironment of protein side chains. In R. J. H. Clark & R. E. Hester (Eds.), *Spectroscopy of biological systems* (pp. 47–112). New York: Wiley.

Voutsinas, L. P., & Nakai, S. J. (1979). Covalent binding of methionine and tryptophan to soy protein. *Journal of Food Science, 44*(4), 1205–1211.

Wong, H. W. (2006). Study of chemically modified food proteins by vibrational spectroscopy. PhD dissertation, The University of Hong Kong.

Yamashita, M., Arai, S., & Fujimaki, M. (1976). A low-phenylalanine, high-tyrosine plastein as an acceptable dietetic food. Method of preparation by use of enzymatic protein hydrolysis and resynthesis. *Journal of Food Science, 41*(5), 1029–1032.

Yamashita, M., Arai, S., Tsai, S. J., & Fujimaki, M. (1970). Supplementing S-containing amino acids by plastein reaction. *Agricultural and Biological Chemistry, 34*(10), 1593–1596.

Zhao, Y., Ma, C.-Y., Yuen, S. N., & Phillips, D. L. (2004a). Study of succinylated food proteins by Raman spectroscopy. *Journal of Agricultural and Food Chemistry, 52*(7), 1815–1823.

Zhao, Y., Ma, C.-Y., Yuen, S. N., & Phillips, D. L. (2004b). Study of acetylated food proteins by Raman spectroscopy. *Journal of Food Science, 69*(3), 206–213.