JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Structural Investigation of Edible Zein Films/Coatings and Directly Determining Their Thickness by FT-Raman Spectroscopy

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Near-infrared Fourier transform Raman (FT-Raman) spectroscopy was employed to study the molecular structure of edible zein films/coatings, which were fabricated directly from zein protein. The secondary structure of zein protein was mainly in α-helix and remained unaltered during film formation as evidenced by the vibrational modes of amide I at 1656 cm^{-1} and amide III at 1274 cm^{-1}. Raman results indicated that hydrophobic interaction played an important role in the formation of zein film and disulfide bonding might be responsible for the structural stability of zein protein during film formation. To enhance its antimicrobial property, an antimicrobial zein film was manufactured by incorporating zein protein with benzoic acid whose structure was then characterized by FT-Raman. It showed that physical entrapment or hydrophobic interaction was crucial to the incorporation of benzoic acid with zein protein, and the secondary structure of the antimicrobial film was still maintained in α-helical form. In addition, FT-Raman exhibits its preference in directly determining the thickness of zein films/coatings. By correlating the Raman intensity ratio of ν_{1003} to ν_{84} ($I_{1003/84}$) versus the thickness of zein film, a linear relationship with high coefficient ($R^2 = 0.9927$) was obtained, which was then used pragmatically to determine the thickness of zein coatings on apple. It showed that the FT-Raman result (thickness = 0.27 ± 0.01 mm) was consistent with that of classical micrometric measurement (thickness = 0.28 ± 0.02 mm). Consequently, FT-Raman provides a direct, simple, and reagent-free method to characterize the structure and the thickness of zein films/coatings.

KEYWORDS: FT-Raman; edible zein films/coatings; zein protein; antimicrobial film; film thickness

INTRODUCTION

Method to protect food from spoilage is an important issue in food industry. The factor causing food spoilage comes mainly from microbial contamination on the food surface (*1*−*3*). To inhibit microbial growth on the food surface, many techniques such as dipping, dusting, and spraying are developed to apply preservatives (antimicrobial agents) to the food surface (*4*−*6*). However, these methods are relatively laborious, and it is difficult to quantify the preservative content resided on food surface. Hence, edible films/coatings from biological materials are then developed for potential uses in food protection and preservation. The development of biopolymers for food packaging and other applications draws much interest due to the foreseeable environmental benefits. Among them, zein film has received worldwide attention because of its biodegradable property and hydrophobic characteristics, which function well as selective barriers to transport of gases, water vapor, or solutes (*7*−*10*). It appears that macroscopic properties of zein-based biodegradable films, including mechanical properties, water absorption, and barrier properties, depend on their three-dimensional network structure and on the interaction between proteins, plasticizers, and other functional agents (*11*). Spectrometric studies indicate that the secondary structure of zein in solution was 50−60% in α-helix, 15% in β-sheet, and the remainder of the molecule is aperiodic (*11*, *12*), while Tatham et al. report that α-helix predominates in the central domains of zein (*13*). Apparently, elucidation of zein film structure, particularly relating to film processing, remains to be clarified.

To ameliorate the contamination problems of food surface, an incorporation method that combines antimicrobials with film material has recently been developed to strengthen the antimicrobial ability of food films (*14*−*18*). It is believed that the quantity and homogeneity of antimicrobial agents in the film and the thickness of film will affect the antimicrobial activity. Thus, characterizing antimicrobial edible film becomes of great importance. Generally, the structural characterization of antimicrobial edible film is carried out by chemical analysis, X-ray, FT-IR, etc. (*19*, *20*). For chemical analysis, it is an indirect method and has to endure several tedious separation steps as well as environmentally unfriendly chemical usage, which will

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10.1021/jf0501490 CCC: $30.25 © 2005 American Chemical Society
Published on Web 06/02/2005

create serious pollution problems. The development of spectroscopic methods such as X-ray (*19*) and FT-IR (*20*) enlightens a new feature of rapidity and efficiency in structural characterization; however, each kind of spectroscopy exhibits itself some shortcomings as used in the study of edible films. For example, X-ray, although powerful in structural interpretation, suffers from high frequency penetration, which usually damages food quality. FT-IR, being mild to foodstuff due to its lower energy characteristics, suffers from sample preparation. Particularly, it is not eligible for water-rich compounds due to its strong absorption of O−H vibrational band. Recently, confocal Raman microspectrometry (CRM) and surface enhanced Raman scattering (SERS) have been used to analyze thin film coatings on poly(ethylene terephthalate) (*21*−*23*). Although CRM and SERS receive promising results on thin film characterization, there remains little Raman-related study of edible zein films so far. In both CRM and SERS methods, samples are excited by a visible light source of which Raman signal is readily overwhelmed by the strong fluorescence interference intrinsically arising from foodstuffs or impurity. The intrinsic yellowish pigmentation of zein protein makes it difficult to use in both methods in zein study. Recently, FT-Raman has been successfully used to detect food borne microorganisms on food surface (*23*). The advantages of FT-Raman spectroscopy in food-related study can be categorized as follows: (1) it is free from fluorescence interference, (2) it has less photodecomposition as compared to classical dispersive Raman measurement, (3) it allows direct sample measurement with no sample destruction, and (4) there is no consuming of unfriendly reagents (*24*). Here, we present FT-Raman study on the structure of zein films/coatings with and without incorporation of benzoic acid. We also report, for the first time, the FT-Raman application in the determination of the thickness of edible zein coatings or films. It is well-known that the thickness is closely related to the property and function of edible zein films/coatings as a food protecting agent. Generally, the thickness of edible films is determined by micrometer. Although direct metric measurement has been used most frequently for thickness determination due to its simplicity and directness, it cannot be used as a direct method of thickness determination for the films/coatings directly onto the food surface. Here, FT-Raman shows its superiority in structural characterization and direct thickness determination of zein coverings/coatings.

MATERIALS AND METHODS

Zein, phosphoric acid, and acetonitrile were ordered from Sigma Chemicals (Sigma Chem. Co., St Louis, MO). Propylene glycol and benzoic acid were from Hayashi Pure Chemical Industries Ltd. (Hayashi Pure Chemical Industries Ltd., Japan). Alcohol was obtained from Taiwan Wine & Tobacco Co. (Chiayi, Taiwan). Phosphoric acid and acetonitrile are HPLC grade, while other reagents except zein are of analytical grade.

Preparation of Edible Zein Films and Their Thickness Determination. Fabrication of edible zein films was carried out by following previous studies (*11*−*13*) with some modifications. Briefly, various thicknesses of cast films were obtained by dissolving zein protein at different concentrations (8%, 10%, 12%, 14%, 16%, and 18%) in 25 mL of 95% alcohol solution containing 2% propylene glycol as plasticizer. An aliquot of 2.0 mL of zein solution was pipetted and put onto a Petri dish for drying. After drying, zein films formed spontaneously and were peeled off from the plate surface. The thickness of zein films was measured at 4−6 spots with a micrometer (SM-112, Teclock, Japan). For fabricating antimicrobial zein film, the above procedures were used except that the antimicrobial agent (benzoic acid) was added into various zein solutions. For example, 2.0 mL of 500 ppm benzoic acid was added into different volumes (30, 40, 50, 60, and 70 mL) of 18% zein solution. After well mixing, aliquots of 5 mL of mixed solution were transferred to a Petri dish for film formation.

FT-Raman Measurement. FT-Raman measurements were performed by using a Bruker RFS-100 FT-spectrophotometer (Bruker Optik GmbH, Lubeck, Germany). Zein film was put onto a stainless steel holder for Raman measurement. Continuous wave near-infrared excitation at 1064 nm was provided by a diode laser pumped Nd:YAG laser (Coherent Lubeck GmbH, Lubeck, Germany). The laser light with power of 150 mW was introduced and focused on the sample. The scattered radiation was collected at 180° with an ellipsoidal mirror and was filtered, modulated, and reflected back into the highly sensitive GaAs detector, which was cooled by liquid nitrogen. Raman spectra were produced over the Raman shift 0−3500 cm^{-1}. Typically, 1000 interferograms were co-added at 4 cm^{-1} resolution with a sampling time of about 30 min. The intensity ratio of Raman bands 1003 cm^{-1} to 84 cm^{-1} ($I_{1003/84}$) was used to evaluate the thickness of zein film. A correlative plot of the Raman intensity ratio ($I_{1003/84}$) versus the thickness of zein films measured by micrometer was established. Each numerical calculation of the Raman intensity ratio was based on the average of triplicate measurements at least.

A practical application of zein protein as edible food coatings was carried out by dipping apples into 18% (w/v) zein solution. After the completion of coatings, the laser line was introduced and shone directly onto the surface of the sample and the thickness of the zein coatings was analyzed by the FT-Raman spectrometer according to the simple linear regression on the calibration plot described above. To confirm the feasibility of the FT-Raman technique in thickness determination, the zein coatings on the apples were skillfully peeled off and the thickness was determined by both the FT-Raman spectrometer and the thickness micrometer for comparison.

Quantification of Benzoic Acid in Zein Film by HPLC. Analysis of benzoic acid was carried out on a PRP-1 reversed phase 100A HPLC column (15 cm × 4.1 mm, 5 μm) (Hamilton Co., Reno, NV) loaded on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Tokoy, Japan). The system was equipped with a model L-7100 pump, a model L-7420 UV−vis detector, and a Rheodyne model 7725 injector. Peaks were detected at wavelength 230 nm, and acquisition and processing of data were completed by Hitachi B-7000 software with AID interface. Mobile phase was prepared by mixing 10 mM phosphoric acid (pH 2.3) and acetonitrile at a ratio of 2:3. Buffer solution was degassed with a Branson 2510 ultrasonic system (Branson Ultrasonic Corporation, Danbury, CT) right before employing. Each sample (1 mg/mL) was filtered with 0.45 μm sterile units (Millipore, Bedford, MA), and 20 μL was injected in the chromatographic system with an optimal flow rate. A typical analysis could be completed in 15 min with the flow rate of 1.0 mL/min.

To study the content of benzoic acid in zein films, about 1.0 g of zein films sampled from three to four batches of films prepared by the same treatment was cut into pieces and suspended in 100 mL of 10 mM phosphate buffer solution (pH 3.5) with stirring. Portions of the solution were sampled, filtered, and analyzed by the HPLC method described before. The quantification of benzoic acid in antimicrobial zein film was accomplished by a simple linear regression on the calibration curve ($R^2 = 0.9996$), which was constructed according to the quantitative determination of standard benzoic acid by HPLC method. The standard calibration curve was set up by plotting the peak area versus the concentration of benzoic acid standards. Data analysis was performed via the SAS system (Copyright 1999−2001 by SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Structural Analysis of Zein Protein and Films. With a simple, direct, and noninvasive measurement, Raman spectroscopy provides a novel method to determine the secondary structure of protein and disclose the local information of protein side groups such as tryptophan, tyrosine, phenylalanine, and sulfhydryl compounds (*25*−*29*). Assignments of characteristic Raman bands were carried out based on previous studies (*25*, *28*) and are listed in **Table 1**. Apparently, the fluctuation of

Table 1. Typical Wavenumbers of Raman Bands and General Assignments in Protein Structure[a]

origin	wavenumber	assignment	structural information
cystine	510	S–S stretch	presence of gauche-gauche-gauche conformation; band broadening and/or shifts may indicate conformational heterogeneity of cystine residues
	525	S–S stretch	gauche-gauche-trans conformation
	545	S–S stretch	trans-gauche-trans conformation
	630–670	C–S stretch	gauche conformation
	700–745	C–S stretch	trans conformation
	2550–2580	S–H stretch	presence of thiol of cysteine residues
tyrosine	850/830	Fermi resonance between ring fundamental and overtone	state of phenolic OH group (exposed or buried, hydrogen-bond donor or acceptor)
tryptophan	760, 880, 1360	indole ring	sharp intense band indicates buried residues; sensitive to environment polarity
phenylalanine	1006	ring breathe	conformation insensitive; useful as an internal intensity standard
aliphatic residues	1450, 1465	C–H bending	microenvironment, polarity
	2800–3000	C–H stretching	microenvironment, polarity
amide I	1655 ± 5	amide C=O stretch, N–H wag	α-helix
	1670 ± 3	amide C=O stretch, N–H wag	antiparallel β-sheet
	1665 ± 3	amide C=O stretch, N–H wag	disordered structure (solvated)
	1685	amide C=O stretch, N–H wag	disordered structure (non-hydrogen bonded)
amide III	>1275	N–H in-plane bend, C–N stretch	α-helix
	1235 ± 5 (sharp)	N–H in-plane bend, C–N stretch	antiparallel β-sheet
	1245 ± 4 (broad)	N–H in-plane bend, C–N stretch	disordered structure
	1235	N–H in-plane bend, C–N stretch	disordered structure (non-hydrogen bonded)

[a] Adapted from Tu (*24*) and Li-Chan (*27*).

vibrational modes of amino acids and amide bonds provides abundant molecular information of protein structure. **Figure 1a** and **b** shows the Raman spectra of zein protein and zein film manufactured from the same protein, respectively. Both spectra indicate that the secondary structure of zein protein and film predominates in α-helix as evidenced by the vibrational mode of amide I at 1654 cm^{-1} as well as amide III greater than 1266 cm^{-1}. This is in good agreement with earlier studies on zein structure that the molecular structure of zein protein is in high content of α-helix (*11*−*13*). Tatham et al. suggest that the α-helix in zein protein is not very flexible and is linked by fairly rigid structures (*13*). This fully supports our results that the secondary structure of zein protein is very stable and experiences little change even during the process of film formation. Characteristic Raman bands of 621 and 1003 cm^{-1} are assigned to phenylalanine; 825 and 852 cm^{-1} represent vibrational modes of tyrosine side-chain. Siamwiza et al. (*29*) report that the tyrosine doublet at 850 and 830 cm^{-1} is sensitive to the nature of hydrogen bond of the phenol hydroxyl group. If a tyrosine residue is on the surface of a protein in aqueous solution, the phenolic OH will be simultaneously an acceptor and donor of moderate to weak H-bond and that the doublet intensity ratio ($I_{850/830}$) will be about 1:0.8 (I = 1.25). If the phenolic oxygen is the acceptor atom in a strong H-bond, the intensity ratio will be about 1:0.4 (I = 2.5). If the phenolic hydroxyl is the proton donor in a strong H-bond, the intensity ratio will be 1:2 (I = 0.5). As a result, the weak intensity of the Raman stretching at 852 cm^{-1} as compared to the Raman peak at 825 cm^{-1} of zein protein implies that tyrosine residues in the edible films are in a buried environment or act as proton donors rather than acceptor. As compared to other proteins such as milk protein, meat protein, seed protein, and lens proteins, zein protein has relatively lower content in tryptophan residue as evidenced by the uncommonly weak Raman intensities at 760, 881, and 1360 cm^{-1}, which are assigned to the vibrational stretching of tryptophan residue. This is consistent with the compositional amino acid analysis documented in earlier studies, which indicated that corn protein was deficient in tryptophan residue

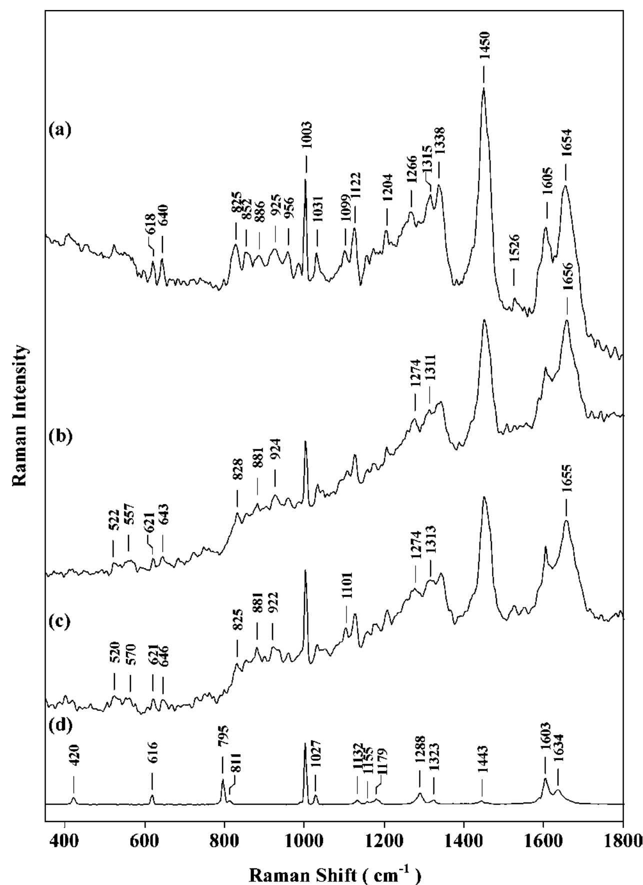

Figure 1. FT-Raman spectra in the region of 350–1800 cm^{-1} for (**a**) zein protein, (**b**) zein protein film without incorporating with benzoic acid, (**c**) zein protein film incorporated with benzoic acid, and (**d**) benzoic acid standard. Conditions for spectrum acquisition are as described in "Materials and Methods".

and lysine (*30*−*32*). The Raman band around 520 cm^{-1} coupling with the lack of Raman stretching in the region of 2500−2700

Table 2. Recovery Ratio of Benzoic Acid from Zein Films

theoretical value (ppm)	HPLC results (ppm)	recovery ratio (%)
1.31	1.29 ± 0.18	98 ± 13.7
1.55	1.66 ± 0.16	107 ± 10.3
1.85	1.84 ± 0.06	99 ± 3.24
2.28	2.35 ± 0.03	103 ± 1.31
3.01	3.10 ± 0.05	103 ± 1.66

cm^{-1} (data not shown) indicates that thiol groups in zein protein are dominated in the oxidation form (S−S bonding). It implies that disulfide bonding plays an important role in maintaining structural stability of zein protein, which is consistent with Paulis's conclusion (*33*). There is a highly spectral similarity between **Figure 1a** and **b** except for 828 and 853 cm^{-1}, which means that tyrosine residues are more sensitive to microenvironmental change during film formation. Similar to zein protein, tyrosine residues in the edible films are in a relatively buried environment or act as hydrogen donors rather than acceptors as shown by the weak intensity of Raman stretching at 853 cm^{-1} in comparison with the band at 828 cm^{-1} (*29*). Reviewing the spectral results revealed by the characteristic amino acids coupling with the extraordinary stability of secondary structure, it implies that hydrophobic interaction plays an important role in the film formation.

Microbial contamination will reduce the shelf life of foods and increase the risk of foodborne illness. Antimicrobial agents such as benzoic acid incorporated into film materials can inhibit microbial growth on the food surface (*14*). The interaction between antimicrobial agents and the film materials is quite interesting and is believed to be critical to the antimicrobial efficiency of the edible films. The mechanism of such antimicrobial interactions remains to be elucidated. FT-Raman is then used to characterize the antimicrobial film structure fabricated by incorporating benzoic acid with zein protein. **Figure 1c** and **d** shows FT-Raman spectra of edible zein films incorporating benzoic acid and benzoic acid standard, respectively. Based on the analysis of FT-Raman spectra of amide I at 1655 cm^{-1} as well as amide III at 1274 cm^{-1}, it clearly reveals that the secondary structure of zein film is unaltered and remains mainly in α-helix as incorporated with benzoic acid. Comparing **Figure 1c** to **b**, there were no significant changes in the Raman profile as benzoic acid was incorporated into zein solution, which implies that no significant chemical bond formed between zein protein and benzoic acid. However, the microenvironment of tyrosine is affected by the incorporation of benzoic acid as shown by the change of Raman intensity of 857 and 828 cm^{-1}. This suggests that physical entrapment or hydrophobic interaction may play an important role in the incorporation of benzoic acid with zein protein. For an antimicrobial film to be effective against microbial growth, the antimicrobial agent should be able to diffuse from packaging material into the food system. Based on the HPLC analysis, more than 95% benzoic acid could be recovered from antimicrobial zein films as shown in **Table 2**. The high recovery ratio of benzoic acid from antimicrobial zein films further supports Raman results that physical entrapment or hydrophobic interaction between benzoic acid and zein protein may serve the main incorporating mechanism in film formation.

Thickness Determination of Zein Coatings or Zein Films. The performance of edible zein films/coatings depends on their composition and morphology. Processing with different thicknesses may affect their composition and morphology, which in turn affects the mechanical and barrier properties of zein films/coatings. **Figure 2** shows the FT-Raman spectra of zein films

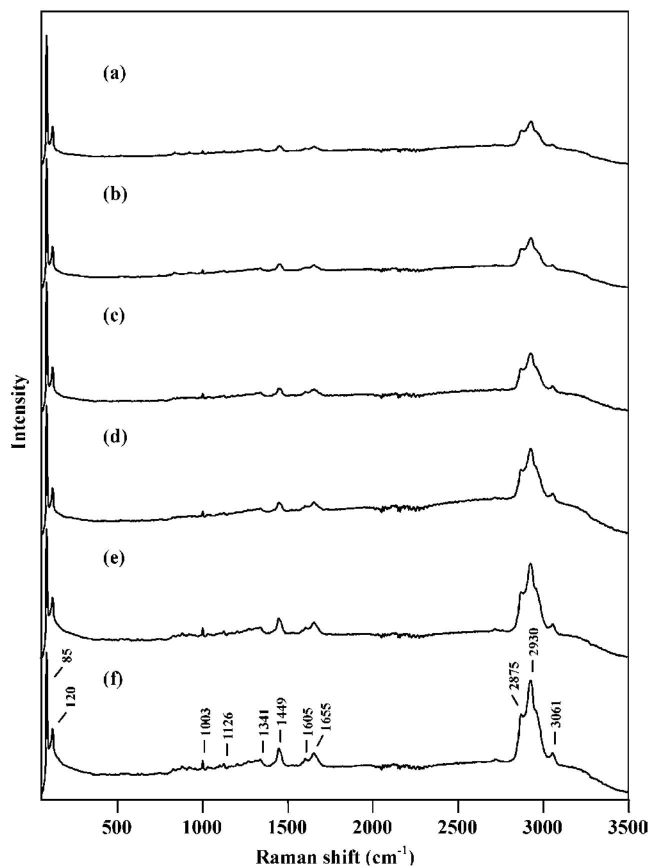

Figure 2. FT-Raman spectra in the region of 50–3500 cm^{-1} for different concentrations of zein films: (**a**) 8%, (**b**) 10%, (**c**) 12%, (**d**) 14%, (**e**) 16%, and (**f**) 18%. Conditions for spectrum acquisition are as described in "Materials and Methods".

manufactured from different concentrations of zein proteins, which results in different thicknesses of zein films. There is no significant change of Raman profiles among different thicknesses of zein films except for the proportional enhancement in the intensity of some Raman bands. This further confirms that physical interactions instead of chemical reactions are the main driving force for the film formation. The intensity of characteristic Raman bands such as 1003, 1449, and 2930 cm^{-1} clearly reflects the increase of zein concentrations or the thickness of zein films. According to earlier studies, the Raman peak of 1003 cm^{-1} due to ring breathe is insensitive to conformational change and is useful as an internal intensity standard (*28*). The intensity of 1003 cm^{-1} shown here is, however, proportional to the thickness of zein films. Thus, the Raman band of 1003 cm^{-1} may be used as an indicator of thickness evaluation. In contrast, the intensity of Raman bands at 84 cm^{-1} is relatively inert to the thickness change of zein films, although their assignments remain unclear. It may be used as an internal reference for evaluating the thickness of zein films. As shown in the inset of **Figure 3**, which displayed the FT-Raman spectra in the region of 50−1100 cm^{-1}, and the intensity normalized at 84 cm^{-1}, it explicitly demonstrated the proportional relationship between the Raman intensity of 1003 cm^{-1} and the thickness of zein films. Therefore, the intensity ratio of ν_{1003} to ν_{84} is used to evaluate the thickness of zein films fabricated from various concentrations of zein proteins. A linear correlation with high coefficient ($R^2 = 0.9927$) between the Raman intensity ratio ($I_{1003/84}$) and the thickness of zein films is obtained, as depicted in **Figure 3**. This illustrates that FT-Raman spectroscopy is able to provide a direct and easy way

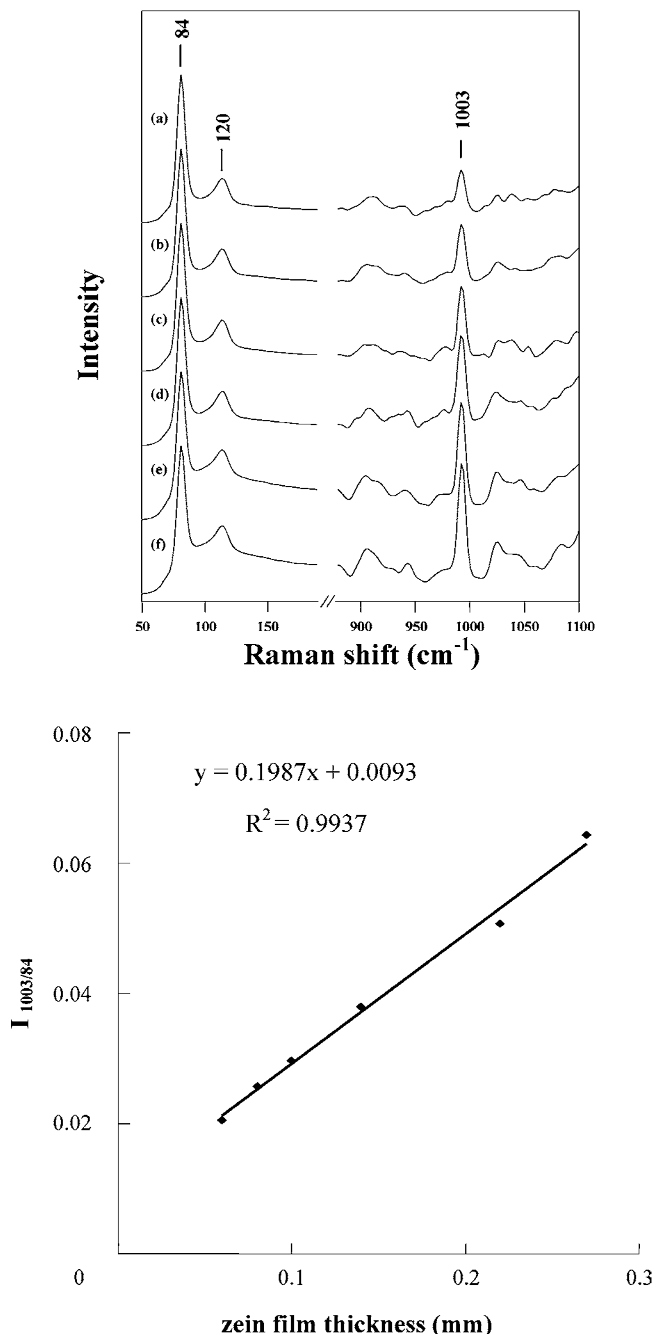

Figure 3. Correlation of Raman intensity ratio of $I_{1003/84}$ versus zein film thickness measured by micrometer. Inset: FT-Raman spectra normalized at the band of 84 cm^{-1} for different concentration of zein films: (**a**) 8%, (**b**) 10%, (**c**) 12%, (**d**) 14%, (**e**) 16%, and (**f**) 18%. Spectral intensity between 900 and 1100 cm^{-1} was enlarged at the same proportion to observe easily the change of Raman intensity upon different zein thicknesses. Conditions for spectrum acquisition are as described in "Materials and Methods".

to determine the thickness of edible zein films. Although the micrometer is usually used to determine films thickness, it cannot be used to determine the thickness of food coatings/coverings. Here, we show a practical application of FT-Raman on the determination of the thickness of food coatings. Zein coatings are completed by dipping apples into a zein protein solution as described in the section of "Materials and Methods". Generally, apples are coated with Shellac and Carnauba wax to improve its appearance, to prevent water loss, and to maintain quality through delayed ripening and senescence. However, both

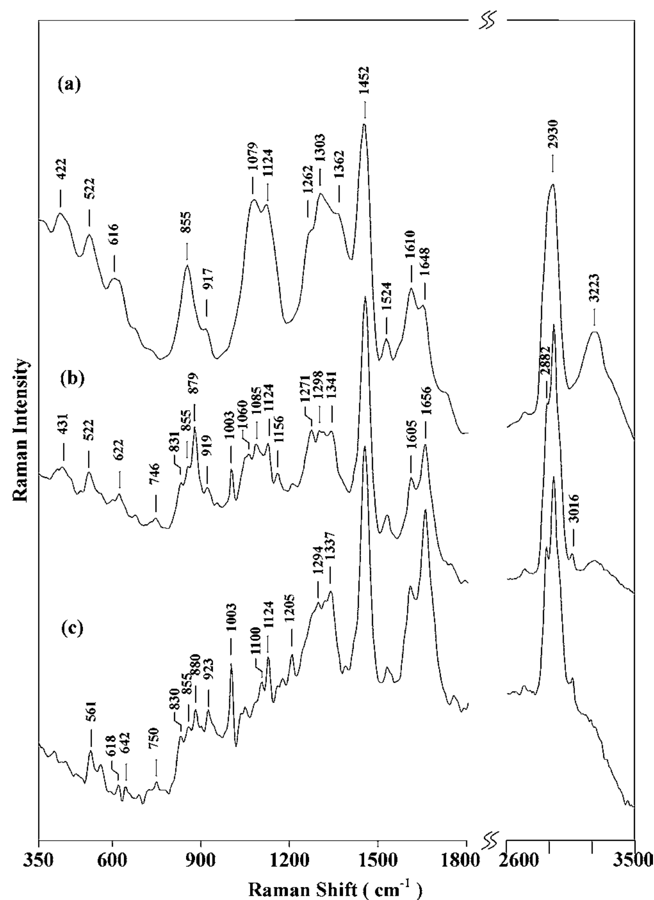

Figure 4. FT-Raman spectra in the region of 350–3500 cm^{-1} for (**a**) apple skin, (**b**) zein film coated onto apple skin, and (**c**) coated zein film after being peeled off from apple skin. Conditions for spectrum acquisition are as described in "Materials and Methods".

materials are associated with nonfood uses (*10*). Zein coatings, on the other hand, have been used to coat nuts and candy for increased gloss, prevention of oxidation, and development of off-odors. The hydrophobic nature of zein, water solubility, and water sorption are lower than or similar to those of other protein and cellulose films (*34*, *35*). Previous studies on the air permeability of zein coatings also show that oxygen and carbon dioxide transmission rates are lower than those of low-density polyethylene (LDPE) as well as cellulose-made films and similar to those of polyester films at the same testing temperature and relative humidity (*36*, *37*). Bai et al. (*10*) report that CO_2 and O_2 permeability of zein coatings are similar to a typical Shellac coating. According to their results of zein coatings for apple, zein coatings are capable of maintaining apple quality similar to a commercial Shellac formulation, and an extended apple shelf life can be obtained as compared to noncoated controls or commercial Carnaubx-coated fruit. Zein coatings thus provide a reasonable alternative to Shellac and Carnauba wax. In **Figure 4**, we demonstrate FT-Raman study of zein coatings on apples. **Figure 4a** is the FT-Raman spectrum of apple skin as laser is introduced and focused on the surface; **Figure 4b** represents the spectrum of zein coatings directly onto apple surface whose preparation is described in the "Materials and Methods section", while **Figure 4c** is the Raman spectrum of the zein coatings after being peeled off from the apple. Comparing **Figure 4a** to **b** and **Figure 4a** to **c**, the remarkable spectral difference between them is anticipated because the composition of apple surface is different from that of zein coatings, which is made up of zein proteins. Raman profile of apple surface (**Figure 4a**) reveals

the composite structure of apple skin as evidenced by the characteristic Raman bands of 855, 1079, 1124, 1262, 1303, 1610, and 1648 cm^{-1}. Although the precise compositional interpretation of the composite structure of apple surface needs to be further elucidated, FT-Raman, however, demonstrates directly the pattern of the surface structure of apple, which is a mixture of carbohydrate, lipid, and proteins. This also shows FT-Raman's distinguished feature for its direct application in food-related study, as compared to the conventional Raman with a laser source of visible wavelength in which Raman signal is usually overwhelmed by fluorescence interference. Characteristic Raman bands such as 522, 622, 642, 830, 855, 1003, 1452, 1605, and 1656 cm^{-1} in **Figure 4b** and **c**, which reveal a typical structural information of zein protein, prove the structural and material consistency of zein coatings before and after being peeled off from apple skin. Raman bands at 2882 and 2930 cm^{-1} are due to C−H stretching, while the band at 3223 cm^{-1} is the vibrational mode of O−H bond. This further supports our discussion above that zein film, either being coated on or being peeled off, can be easily differentiated from apple skin by FT-Raman, and FT-Raman shows great potential for simply and directly determining the thickness of food coatings or packing films. Based on the linear regression analysis, by using the correlative diagram of **Figure 3**, the thickness of both zein coatings, before and after being peeled off, is determined to be 0.27 ± 0.01 mm. This is very close to the one determined by thickness micrometer, 0.28 ± 0.02 mm. As mentioned before, thickness micrometer cannot be used for the thickness determination of edible films or covering/coatings direct on food surface. In contrast, FT-Raman method is able to directly determine the thickness of zein coatings/films.

In conclusion, FT-Raman spectroscopy demonstrates itself as a powerful tool to be applied in the study of zein protein and films. Being free from fluorescence interference as well as free from pollutant chemicals usage, FT-Raman can be either qualitatively characterizing the molecular structure of zein films, with or without incorporating antimicrobial agent (benzoic acid), or quantitatively determining the thickness of zein coatings/films. Based on the FT-Raman results, it shows that the secondary structure of zein protein is mainly in α-helical form and is uncommonly stable during film formation. As demonstrated by the characteristic Raman bands of major amino acids, physical entrapment or hydrophobic interaction plays an important role in the incorporation of benzoic acid with zein protein, and disulfide bonding is crucial to maintaining structural stability of zein protein. The Raman intensity ratio of $I_{1003/84}$ can be applied to determine the thickness of zein coatings or film coverings directly upon food products. In short, in this investigation, near-infrared Raman spectroscopy proves itself to be a powerful technique in qualitatively characterizing film structure and quantitatively determining the thickness of zein films/coatings.

ACKNOWLEDGMENT

We thank for Prof. Mong Liang (National Chiayi University) for his assistance in reading this manuscript.

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Received for review January 20, 2005. Revised manuscript received April 11, 2005. Accepted April 30, 2005. Financial support (NSC-92-2214-E-415-003 and NSC-92-2113-M-415-008) from the National Science Council of Taiwan is much appreciated.

JF0501490