Study of Succinylated Food Proteins by Raman Spectroscopy

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Three food protein products, soy protein isolates, spray-dried egg white, and whey protein isolates, were chemically modified to varying levels with succinic anhydride, and the extent of modification of these proteins were determined by standard wet chemistry methods. Raman spectra (500–2000 cm^{-1}) of the modified proteins were obtained. New C=O stretching vibrations were observed at 1420 and 1737 cm^{-1} and were attributed to the carboxylate (COO$^-$) and ester carbonyl (RCOO-) groups, respectively, which were appended to the proteins during succinylation. Two series of calibration curves were obtained by plotting the intensity ratio of the 1420 and 1737 cm^{-1} to 1003 cm^{-1} phenylalanine stretching band (used as an internal standard) against the extent of substituted ϵ-amino (and α-amino) groups and aliphatic hydroxyl groups, respectively. Linear fits were obtained with correlation coefficient $r > 0.988$. The Raman spectral data were also analyzed to study the effect of succinylation on the conformation of the three proteins. Some conformational changes were observed, including a transition from ordered to disordered structures, an exposure of tryptophan residues from a buried, hydrophobic microenvironment, and probably conformational shift of cystine residues.

KEYWORDS: Succinylation; proteins; soy protein isolates; egg white; whey protein isolates; Raman spectroscopy

INTRODUCTION

Because many food proteins in their native state do not necessarily possess optimal nutritional and functional properties, chemical modification of proteins has been extensively studied and has been shown to be a very powerful tool for both improving the functional properties (*1*, *2*) and studying the structure–function relationships of these biomolecules (*3*). When compared to other procedures such as physical methods and genetic engineering, chemical modification has the advantage in that the modified proteins are easy to prepare in a large scale from commercial materials (*4*). One of the most common chemical modifications used for proteins is the acylation of amino acid residues with acid anhydrides. Most studies related to food proteins have been reported on modification with succinic anhydride (*5*). This reagent does not react selectively with one type of functional groups but reacts with all nucleophilic groups (*6*). These include amino groups (N-terminal, α-, and lysine ε-amino groups), phenolic (tyrosine) and aliphatic (serine and threonine) hydroxyl groups, sulfhydryl groups (cysteine), and imidazole (histidine) groups. Both the reactions of these groups and the stability of their acyl derivatives differ considerably. The ε-amino group of lysine is the most readily acylated group due to its relatively high reactivity and steric

availability for reaction (*5*). The succinylation of hydroxyl amino acids occurs at a sufficiently high excess of the reagent.

The increase in negative charge of proteins caused by introducing additional carboxyl groups may result in drastic changes in the native conformation and functional properties (*5*). The extent of modification of the functional groups of a protein can be varied widely by changing the amount of the acylating agent. The related changes in functional and physicochemical properties of food proteins are influenced by the degree of modification (*7*). Therefore, it is important to quantitatively control the desired degree of acylation of a protein for different applications. Traditionally, the degree of acylation in modified proteins is determined using wet chemistry techniques (*8*, *9*). These methods are destructive, involve time-consuming sample preparation procedures, and require a relatively large amount of samples. The difficulty in dissolving vegetable proteins is one of the main reasons for lengthy sample preparation in working with these materials. Thus, it would be most convenient to select an analytical method that could work with solid protein samples and provide a direct, nondestructive, and fast determination of the degree of acylation in modified proteins.

Recently, Phillips and co-workers (*10*, *11*) developed an analytical method using Raman spectroscopy for the determination of degree of acetylation and succinylation in modified starches. In contrast to many other spectroscopic methods, Raman spectroscopy has a distinct advantage in not requiring optically clear solutions. It has been used to study molecules in aqueous solutions or solids, which can be analyzed directly in

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air, at ambient temperature and pressure, wet or dry, and in many cases without destroying the sample. The intensity of a Raman band contributing to a Raman spectrum depends linearly on the amount of substance contributing to that Raman band (*12*, *13*). Both qualitative and quantitative information can be obtained by Raman spectroscopy. The Raman peak positions and relative intensities are very sensitive to the structure of the molecule and the environment surrounding that molecule. Raman spectroscopy has been used in the study of food proteins (*14−17*). A review by Li-Chan (*18*) indicated that Raman spectroscopy can be used for rapid quality control as well as to provide a wealth of detailed in situ structural information on food systems under conditions relevant to processing. The main purpose of succinylation of food proteins is to improve functional properties attributed to change in physicochemical characteristics resulting from altered protein conformation. Hence, it is important to study the effect of acylation on the conformation of proteins.

In this investigation, the development and application of Raman spectroscopy to determine the extent of succinylation in chemically modified soy protein isolates, whey protein isolates, and egg white proteins will be described. The effect of succinylation on the conformation of these proteins will also be studied by Raman spectroscopy.

MATERIALS AND METHODS

Materials. Commercial SPI, Supro 610, was obtained from Protein Technology International Co. (St. Louis, MO). Spray-dried EW were provided by the Canadian Inovatech Inc. (Abbotsford, BC, Canada). WPI were obtained from Foremost Farms (Waukon, Iowa, Darietak NVB 389, Lot #21-4080, containing 89.43% protein). Succinic anhydride, L-lysine monohydrochloride, 2,4,6-trinitrobenzenesulfonic acid (TNBS), and hydroxylamine hydrochloride were from Sigma Chemical Co. (St. Louis, MO). Monomethyl succinate was from Aldrich Chemical Co., Milwaukee, WI.

Succinylation. Protein samples were succinylated according to the procedure described by Franzen and Kinsella (*19*) with some modifications. Samples (2 g) of protein were dispersed in approximately 80 mL of distilled water to make up 2.5% dispersion with 1 h of magnetic stirring. The pH of the dispersions was adjusted to about 8.0 by adding 2 N NaOH. Succinylation was accomplished by the addition of small amounts of solid anhydride to rapidly stirred protein dispersions. The pH was maintained between 7.5 and 8.5 by the addition 2 N NaOH. After the pH was stabilized at about 8.0 following the addition of succinic anhydride, it was kept at about 8.0 for another 1 h for the reaction to go to completion. After that, the pH was decreased to 7.0 with the addition of 1 N HCl to prevent further modification. A control was prepared by the same procedure without addition of succinic anhydride. The solutions were dialyzed at 4 °C for 48 h to remove impurities and excess reagents, and protein samples were recovered by freeze-drying.

Determination of Succinylated Lysine. Succinylated lysine in modified protein samples was quantified by determining the available lysine following the method described by Hall (*8*). Standard 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution was added to a suspension of protein sample and held for 75 min in a 40 °C water bath, followed by 2 h of heating in a boiling water bath. Absorbance of the ϵ-TNP-lysine released by hydrolysis of the reaction mixture was measured at 415 nm. The amount of available lysine was determined from a standard curve. The extent of modification was expressed as percentage and was calculated based on the decrease in absorbance because fewer amino groups are available to react with the TNBS reagent. Although both ϵ-amino and α-amino groups react with TNBS, the ϵ-amino groups are 30 times more reactive than the α-amino groups (*20*). Hence, the determined amino groups were mainly attributed to the ϵ-amino groups of lysine.

Determination of Succinylated Hydroxy Amino Acids. The amount of succinylated hydroxy amino acids in modified protein

Table 1. Percentage of Succinylation[a] and Raman Intensity Ratio of SPI, EW, and WPI

| sample	ϵ-NH$_2$[b]	−OH[c]	ϵ-NH$_2$+ −OH	h_{1420}/h_{1003}	h_{1737}/h_{1003}
SPI					
control (A)	0.00	0.00	0.00	0.656	0.000
sample 1 (B)	1.46	0.26	1.72	0.685	0.012
sample 2 (C)	3.94	0.40	4.34	0.720	0.008
sample 3 (D)	5.77	0.90	6.67	0.789	0.015
sample 4 (E)	7.38	1.32	8.70	0.876	0.038
sample 5 (F)	7.40	3.00	10.40	0.948	0.082
sample 6 (G)	7.39	6.02	13.41	1.036	0.144
sample 7 (H)	7.41	7.31	14.72	1.067	0.166
EW					
control (A)	0.00	0.00	0.00	0.508	0.000
sample 1 (B)	2.32	0.00	2.32	0.538	0.003
sample 2 (C)	3.76	0.00	3.76	0.569	0.002
sample 3 (D)	4.87	0.51	5.38	0.610	0.025
sample 4 (E)	7.09	3.47	10.56	0.707	0.057
sample 5 (F)	6.93	4.34	11.44	0.751	0.069
sample 6 (G)	7.10	5.67	12.60	0.796	0.090
sample 7 (H)	7.20	6.91	13.80	0.839	0.107
WPI					
Control (A)	0.00	0.00	0.00	0.782	0.000
sample 1 (B)	2.53	0.22	2.75	0.875	0.012
sample 2 (C)	5.16	0.17	5.33	0.938	0.017
sample 3 (D)	7.10	0.26	7.36	1.001	0.015
sample 4 (E)	8.43	0.62	9.05	1.065	0.020
sample 5 (F)	8.86	1.47	10.33	1.099	0.031
sample 6 (G)	8.54	2.62	11.16	1.155	0.059
sample 7 (H)	8.75	3.64	12.39	1.210	0.075
sample 8 (I)	8.79	4.42	13.11	1.249	0.087

[a] Averages (g /100 g protein) of duplicate determinations [b] Determined by the TNBS method. [c] Determined by the alkaline hydroxylamine method.

samples was measured by the alkaline hydroxylamine method as described by Gounaris and Perlmann (*21*). The reagent consisted of 2 M NH$_2$OH−HCl, 3.5 M NaOH, and 0.05 M borate buffer in the proportions of 2:1:1, v/v/v. An aliquot (2 mL) of this reagent was added to a 1-mL protein solution (5 mg/mL), and the mixture was incubated at 40 °C for 2 h. The reaction was stopped by addition of 1.0 mL of concentrated HCl (diluted 1:3, v /v, with water) followed by 1.0 mL of 0.37 M FeCl$_3$ in 0.1 M HCl. The precipitated protein was removed by centrifugation, and the absorbance of the supernatant was measured at 540 nm 15 min after adding FeCl$_3$. Quantification was based on a standard curve of the reaction of monomethylsuccinate with the alkaline hydroxylamine reagent. The extent of modification was also expressed as percentage and calculated based on decreases in absorbance.

Raman Spectroscopy. Raman spectra were recorded on a Renishaw-Raman Imaging Microscope (system 1000) equipped with a 514 nm Argon ion laser excitation at 100 mW power (Spectra Physics, Mountain View, CA). The laser was focused on the solid samples placed on microscope slides. Each spectrum was obtained under the following conditions: 10 scans, 30-s exposure time, 2-cm^{-1} resolution. The averaged spectral data from the scans of samples in the Raman spectrophotometer were baseline corrected and normalized against the phenylalanine band at 1003 cm^{-1}. The Raman spectra (200−2000 cm^{-1}) of each sample were performed in duplicate and the results were reported as the averages of these replicates (relative standard deviation < 5%).

RESULT AND DISCUSSION

Determination of Extent of Succinylation by Wet Chemistry Methods. In addition to the modification of lysine residues, succinylation may have occurred at other sites of the proteins. *O*-Succinyl tyrosines undergo a spontaneous intramolecularly catalyzed hydrolysis at pH > 5, and *N*-succinyl histidines are also unstable (*6*). The potential modified groups in the three proteins are therefore ϵ-amino groups (lysine), aliphatic hydroxyl groups (serine and threonine), and sulfhydryl groups. Because the S−H stretching bands in 2550−2580 cm^{-1} (*18*, *22*, *23*) were

Succinylation of ε-amino groups
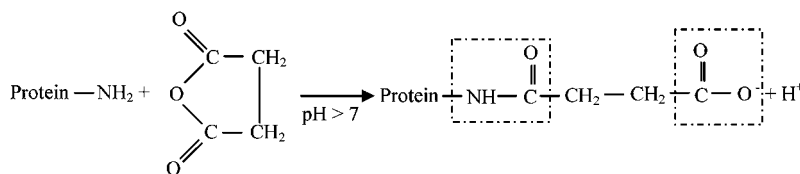
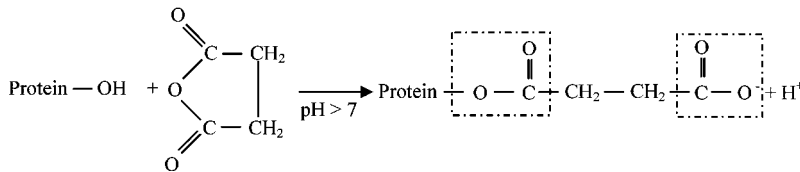**Succinylation of hydroxy amino acids**

Figure 1. Major reactions of succinic anhydride with proteins (adapted from ref 33)

not clearly identified in the Raman spectra of the three protein products, only lysine ϵ-amino groups and aliphatic hydroxyl groups were analyzed in this study.

Table 1 lists the extent of succinylation of the three proteins when reacted with different amounts of succinic anhydride. Succinic anhydride first reacted with lysine, and the quantity of substituted ϵ-amino groups increased with an increase in the level of succinylation, ranging from 18% to 95%. The hydroxyl groups were also succinylated to a lesser extent when un-reacted lysine residues were still present but increased sharply when the reaction with the lysine residues was essentially complete (**Table 1**). These results are in agreement with other reports (*21, 24, 25*), and reflect the lower reactivity of hydroxyl groups to acylation. Succinylation of proteins introduces three additional functional groups: amide ($-CO-NH-$) due to succinylation of ϵ-amino groups of lysine, carboxylate ($-COO^-$ or $-COOH$) released upon reaction of succinic anhydride with the protein functional groups, and ester carbonyl ($ROOC-$) due to the substitution of aliphatic hydroxyl groups (**Figure 1**). The first two groups are also present in the parent proteins, corresponding to the amide groups of peptide bonds and carboxylate groups of aspartic and glutamic acids, but the ester carbonyl is a new group absent in the parent proteins. In Raman spectroscopy, these groups are characterized by the absorption of the C=O stretching frequency located in a broad region from 1400 to 1800 cm^{-1} (*26*).

Determination of Extent of Succinylation by Raman Spectroscopy. The Raman spectra for the three protein samples with increasing percentage of succinylation are shown in **Figures 2–4**. Assignments of some major Raman bands (**Table 2**) were carried out according to previous workers (*18, 22, 23*). The intensity and location of the phenylalanine band at 1003 cm^{-1} band is not sensitive to conformation or to the microenvironment and therefore can be used as an internal standard (*18*). When compared to the unmodified controls, the intensity of several Raman bands changed upon succinylation. We found the 1737 cm^{-1} and the 1420 cm^{-1} Raman bands provided the most convenient marker bands for succinylation. Normally, carboxyl group vibrational bands are not sensitive to conformational changes but are sensitive to the state of ionization. The band at 1400–1430 cm^{-1} could be attributed to the dissociated or ionized carboxyl (COO$^-$) groups while, the protonated form (COOH) exhibits a band at 1700–1750 cm^{-1} (*22*). In this study, all samples were treated at pH 7.0–8.0, and the ionized carboxyl groups may have vibrations at 1400–1430 cm^{-1}. The band observed at 1420 cm^{-1} can therefore be attributed to the C=O bonds of COO$^-$ groups.
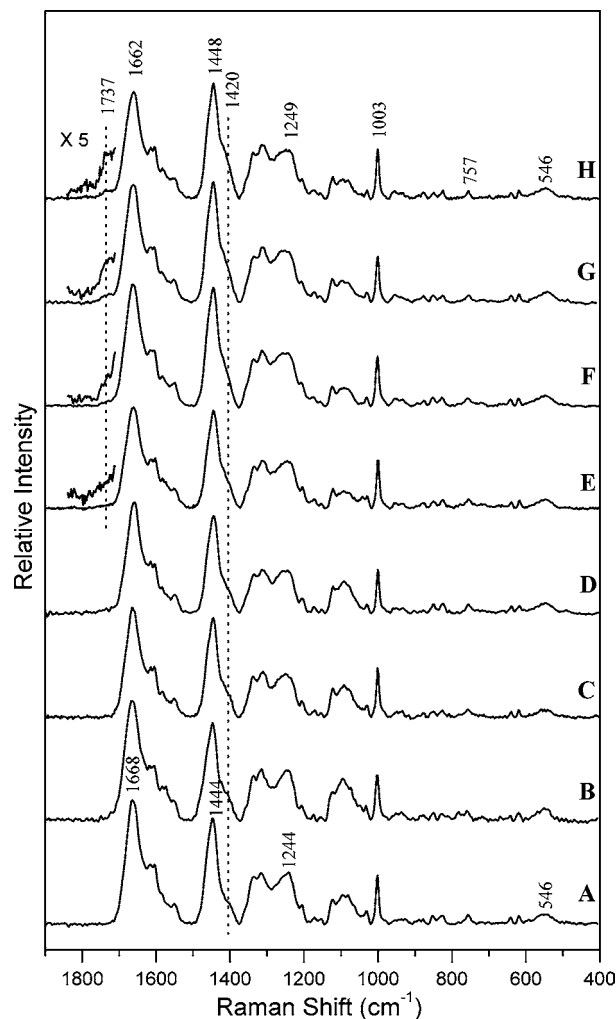
Figure 2. Raman spectra of control (nonsuccinylated) (**A**) and seven succinylated soy protein isolates with increasing percentage of modification (**B–H**).

Table 1 lists the ratio of the intensity of the Raman 1420 cm^{-1} COO^{1-} vibrational bands to the 1003 cm^{-1} phenylalanine bands for each of the three protein samples. The results indicate progressive increases in intensity of the 1420 cm^{-1} band with an increasing percentage of succinylation. **Figure 5** shows the plots of the Raman band ratios against the percentage of succinylation for each of the three proteins. Representative plots, constructed from data obtained from two separate samples of each protein, indicate best fit linear regressions to the data
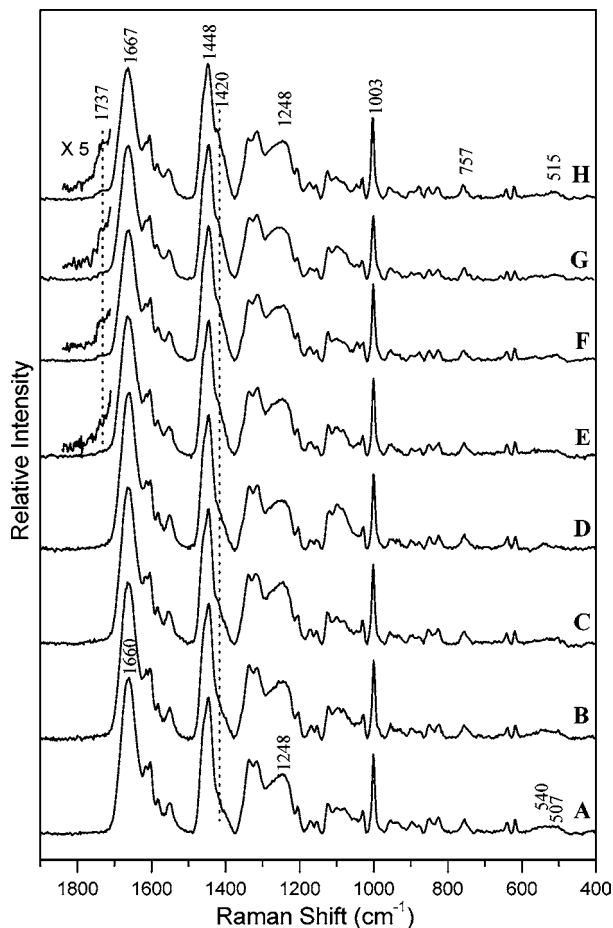
Figure 3. Raman spectra of control (nonsuccinylated) (**A**) and seven succinylated egg white with increasing percentage of modification (**B**–**H**).

plotted in each figure. The calibration equations ($y = B \times X + A$; where y is the ratio of the 1420 cm^{-1} band to 1003 cm^{-1} band, X is the percentage of substitution of ϵ-amino and hydroxyl groups, B is the slope, and A is the y-intercept) for each protein are shown in **Table 3**. The calibration curves have slopes of 0.02724, 0.02364, and 0.03461 for SPI, EW and WPI, respectively, and the y-intercept varied from 0.4879 to 0.7668, with linear regression correlation coefficient of $r \geq 0.9868$ (**Table 3**). The correlation coefficients indicate a strong linear correlation between the I_{1420}/I_{1003} intensity and the percentage
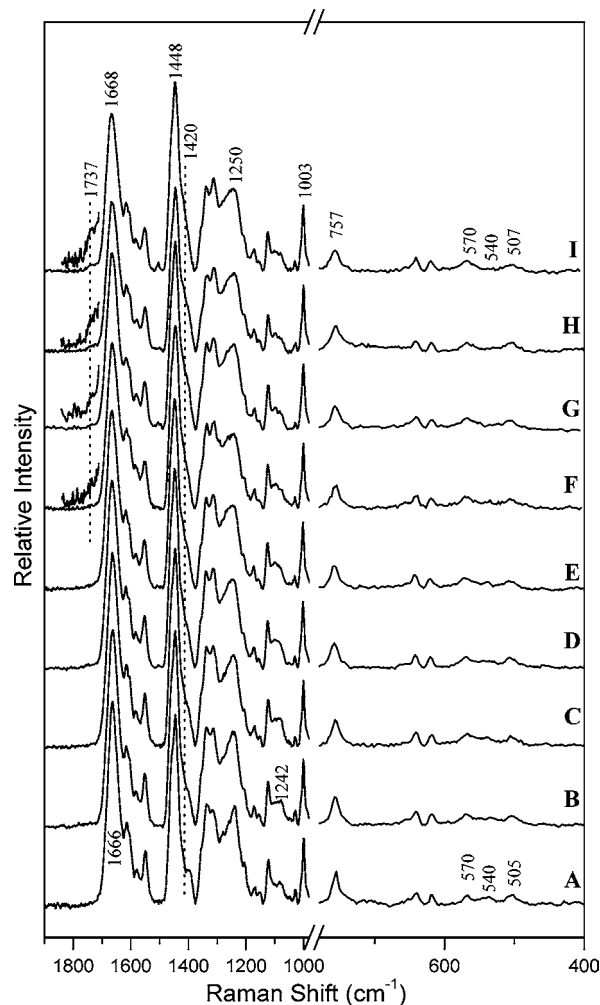

Figure 4. Raman spectra of control (nonsuccinylated) (**A**) and eight succinylated whey protein isolates with increasing percentage of modification (**B**–**I**).

of succinylation for each of the three proteins. We also noted that the marker Raman band has a background from the native proteins. This background was mainly attributed to the carboxyl group vibrations of aspartic and glutamic acids of the parent proteins. When the three sets of data were pooled and plotted,

Table 2. Typical Wavenumbers of Raman Bands and General Assignments in Raman Protein Spectra[a]

origin	wavenumber (cm^{-1})	assignment	structural information
cystine	510	S–S stretch	gauche–gauche-gauche conformation
	525	S–S stretch	gauche–gauche-trans conformation
	545	S–S stretch	trans-gauche–trans conformation
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	1003	ring breathe	conformation insensitive; useful as an internal intensity standard
aspartic and glutamic acids	1400–1430	C=O stretch of COO$^-$	ionized carboxyl groups
aliphatic residues	1450, 1465	C–H bending	microenvironment, polarity
amide I	1650–1660	amide C=O stretch, N–H wag	α-helix
	1667–1673	amide C=O stretch, N–H wag	antiparallel β-sheet
	1665 ± 3	amide C=O stretch, N–H wag	random coil
amide III	1260–1300	N–H in-plane bend, C–N stretch	α-helix
	1230–1240	N–H in-plane bend, C–N stretch	antiparallel β-sheet
	1245 ± 4	N–H in-plane bend, C–N stretch	random coil

[a] Adapted from Li-Chan (*18*), Tu (*21*), and Li-Chan and Nakai (*22*).

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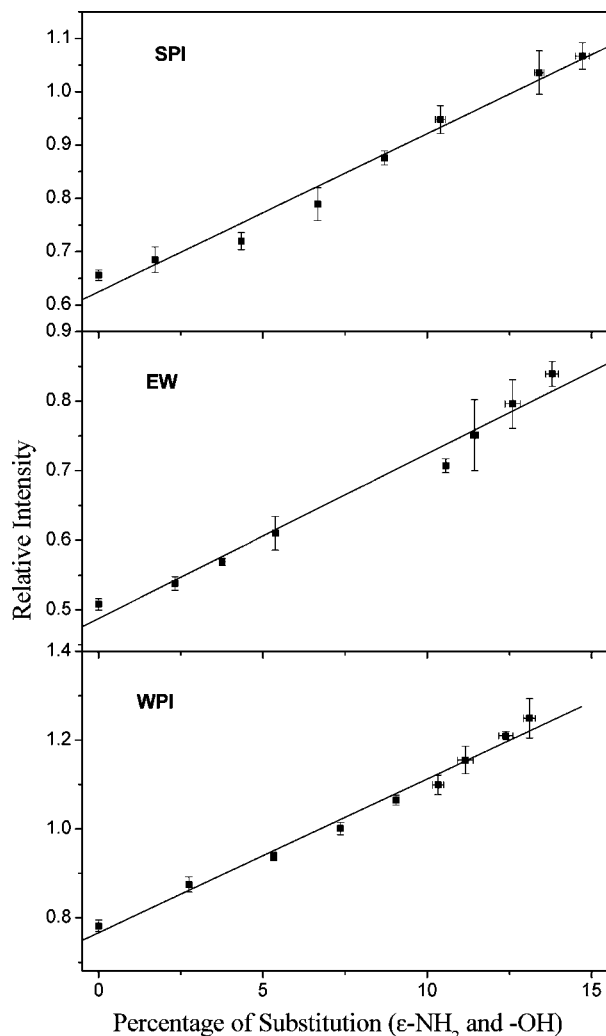*J. Agric. Food Chem.,* Vol. 52, No. 7, 2004 **1819**

Figure 5. Plots of the ratio of Raman 1420 cm^{-1} band to 1003 cm^{-1} band versus the percentage of substitution of ϵ-amino and hydroxyl groups determined by wet chemistry methods. The error bars of the data points represent $\pm$ 1 SD of the Raman (*y*-axis) and wet chemistry (*x*-axis) measurements. The linear regression parameters are summarized in **Table 3**.

Table 3. Parameters for Linear Regression Analysis of the Calibration Curves for the Raman Intensity Ratio vs Percentage of Substitution of ϵ-Amino and Hydroxyl Groups[a,b]

sample	B	A	r
soy protein isolate	0.02724	0.6424	0.9868
egg white	0.02364	0.4879	0.9898
whey protein isolate	0.03461	0.7668	0.9926
SPI + EW + WPI	0.03031	0.6268	0.4669

[a] $y = A + B \times X$; where y is the ratio of the 1420 cm^{-1} band to 1003 cm^{-1} band, X is percentage of substitution of ϵ-amino and hydroxyl groups, B is slope, and A is *y*-intercept. [b] Averages of duplicate determinations. Separate sets of data were fit to different regression equations for individual proteins, and the combined data were used for the regression equation of the three proteins (last row).

the resulting correlation coefficient (0.4669) was markedly decreased, indicating much weaker linear correlation (**Table 3**).

Comparison with the unmodified control spectra revealed that the protein samples at high levels of succinylation showed a new peak at 1737 cm^{-1}. The 1737 cm^{-1} band can be attributed to the C=O bonds of ROOC$-$ groups, which are characterized by an absorption near 1740 cm^{-1} (*27*). The ratios of the Raman
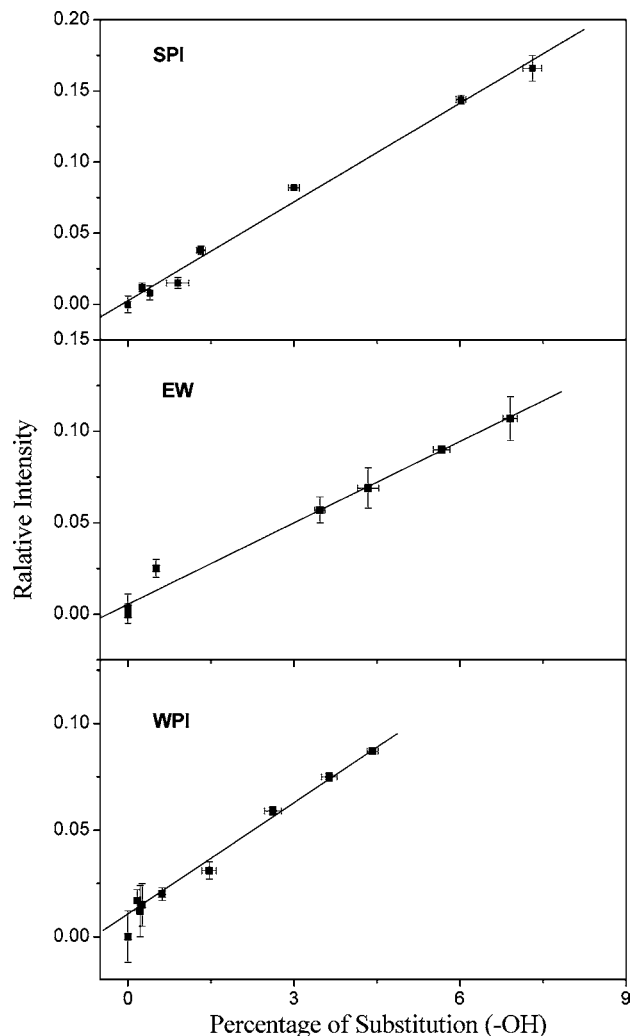

Figure 6. Plots of the ratio of Raman 1737 cm^{-1} band to 1003 cm^{-1} band versus the percentage of substitution of hydroxyl groups determined by the alkaline hydroxylamine method. The error bars of the data points represent $\pm$ 1 SD of the Raman (*y*-axis) and wet chemistry (*x*-axis) measurements. The linear regression parameters are summarized in **Table 4**.

intensity of the 1737 cm^{-1} C=O stretching band to 1003 cm^{-1} phenylalanine band for each of three protein samples were calculated (**Table 1**). The data show a progressive increase in intensity of the 1737 cm^{-1} band with an increase in the percentage of succinylated hydroxyl groups, which did not occur before the completion of the reaction of ϵ-amino groups. The intensity ratio of the 1737 cm^{-1} to 1003 cm^{-1} Raman bands was plotted against the percentage of substitution for hydroxyl groups, and a linear regression line was plotted to the data (**Figure 6**). The calibration equations, constructed from data obtained from two separate samples of each protein, for the three protein samples were also obtained (**Table 4**). The calibration curves have correlation coefficients $r \geq 0.9930$, and their slopes are 0.02253, 0.01556, and 0.01727, for the SPI, EW, and WPI proteins, respectively. These results conclusively demonstrate that the 1737 cm^{-1} band intensity has a strong linear relationship with the percentage of substitution for hydroxyl groups for individual proteins. It was also noted that zero intensity for the control samples (no substitution) was almost within the uncertainty of the linear regression *y*-intercept (ranging from 0.012 to 0.0112), indicating that the 1737 cm^{-1} band is mainly attributed to the succinylation of hydroxyl groups with very

Table 4. Parameters for Linear Regression Analysis of the Calibration Curves for the Raman Intensity Ratio vs Percentage of Substitution of Hydroxyl Groups[a,b]

sample	B	A	r
soy protein isolate	0.02253	0.0065	0.9964
egg white	0.01556	0.0012	0.9994
whey protein isolate	0.01727	0.0112	0.9930
SPI + EW + WPI	0.01859	0.005542	0.9263

[a] $y = A + B \times X$; where y is the ratio of the 1737 cm^{-1} band to 1003 cm^{-1} band, X is percentage of substitution of hydroxyl groups, B is slope, and A is y-intercept. [b] Averages of duplicate determinations. Separate sets of data were fit to different regression equations for individual proteins, and the combined data were used for the regression equation of the three proteins (last row).

small contribution from the parent proteins. When pooled data from the three proteins were plotted, the resulting correlation coefficient (0.9263) was lower than those for individual proteins (**Table 4**), indicating decreased linear relationship.

Because the three kinds of proteins have different chemical structures, the C=O groups added to them by the succinylation process would experience somewhat different molecular environments. The slopes of the calibration lines for the three proteins are different from each other, probably due to different microenvironment during succinylation. The data suggest that, to obtain the most accurate results, we should use a calibration curve for that particular protein system. Using these calibration curves, we can obtain the amounts of succinylated aliphatic hydroxyl groups (serine and threonine) and succinylated ε-amino groups (lysine) from the intensity ratios of the 1737 and 1420 to 1003 cm^{-1} Raman bands, respectively, as well as the sum totals of substituted residues.

The proposed Raman method has several important advantages over other procedures for determining the percentage of succinylation in modified proteins. First, the Raman method requires almost no sample preparation for the solid proteins, which makes the method much more convenient as well as potentially faster. Second, the Raman method is essentially nondestructive, whereas wet chemistry methods are destructive tests and toxic or corrosive reagents may be required. Third, the Raman method can potentially be developed for a quality control protocol for fast analysis of succinylated proteins and even in situ monitoring of the sucinylation process.

Effect of Succinylation on Protein Conformation. (*a*) *Backbone Conformation.* The amide or peptide bond has several distinct vibrational modes. Raman bands corresponding to amide I and amide III can be used to characterize backbone conformation, giving information on the relative proportions of different types of secondary structures in polypeptides or proteins (*18*). Generally, proteins with high α-helical contents show an amide I band centered around 1650–1660 cm^{-1} and a weak amide III band located in a broad region from 1260 to 1300 cm^{-1}. Proteins with predominantly β-sheet structures show amide I band at 1667–1673 cm^{-1} and a more intense amide III band near 1230–1240 cm^{-1}. Proteins containing a high proportion 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} (*18, 22, 28*).

In the unmodified SPI, the amide I and amide III bands were centered at 1668 and 1244 cm^{-1}, respectively, indicating that β-sheets and random coils are the major secondary structures. This is in agreement with the data reported by Achouri and Zhang (*29*), where the secondary structure composition of SPI, measured by circular dichroism spectroscopy, was 35% β-sheets, 10% β-turns, 34% random coils, and 21% α-helices. Raman spectral data (**Figure 2**) show that with an increase in the extent

of succinylation, there was a progressive shift in the amide I vibration to lower wavenumbers, indicating a transition from β-sheet to random coil structures was taking place. There were also shifts in the amide III band from 1244 to 1249 cm^{-1}, and this also suggests a transformation from β-sheet to random coil structures takes place. It has been previously reported that succinylation of SPI led to protein denaturation, dissociation of the oligomeric proteins into subunits, and marked increases in the net charge and electrostatic repulsion causing the molecules to expand and undergo conformational changes (*19, 29*).

The amide I and amide III bands of the unmodified EW were centered at 1660 and 1248 cm^{-1}, respectively (**Figure 3**), in agreement with the data reported by other workers (*30, 31*). The present data in **Figure 3** suggests that α-helix and random coil are the predominant structures. Ovalbumin is the major protein in egg white, and it has a relatively high content of helical structure (*32*). With an increase in the percentage of succinylation, the amide I bands were shifted to higher wavenumbers (from 1660 to 1667 cm^{-1}), and the amide III bands showed no marked changes. These results suggest that succinylation led to a more disordered conformation, corresponding to a transition from α-helical structures to random coil structures.

The amide I and amide III vibrations were located at 1666 and 1242 cm^{-1}, respectively, in the unmodified WPI (**Figure 4**), indicating that β-sheets are the major secondary structures. Howell and Li-Chan (*33*) reported that the amide I band of WPI is centered at a slightly higher wavenumber of 1670 cm^{-1}. It has been shown that β-lactoglobulin, the major protein component in whey, has 40–50% β-structure and less than 10% α-helices (*34*). Upon succinylation, the amide III band of succinylated WPI was shifted to higher wavenumbers (from 1242 to 1250 cm^{-1}), suggesting more random coil formation. No significant shift in amide I vibrations was observed upon succinylation.

It should be noted that new amide bonds were formed upon succinylation of amino groups, and these could also contribute to changes in the amide I and amide III bands, especially increases in intensity. However, based on previous studies that showed marked conformational changes in succinylated proteins (*1, 2, 5, 7*), the observed changes in Raman spectra (particularly shift in band positions) should be contributed, to a large extent, to altered secondary structures in the three modified proteins.

(*b*) *Side Chain Vibrations.* Bands attributed to various stretching (2800–3000 cm^{-1}) or bending (near 1450 cm^{-1}) vibrational modes of the amino acid functional groups can be used to monitor the environment around these side chains (*18, 22, 28*). It has also been suggested that Raman bands of the aromatic amino acid side chains can be used to monitor the polarity of the microenvironment, or involvement in hydrogen bonding (*18*).

The intensity of C−H bending vibration band was increased in the highly succinylated SPI (**Figure 7**), indicating changes in microenvironment and polarity (*18*) and suggesting protein unfolding, similar to that observed in oat globulin (*35*) and red bean globulin (*36*).

A tryptophan band near 757 cm^{-1} was observed in WPI, and the transition intensity decreased progressively upon succinylation (**Figure 7**). These results suggest that tryptophan residues from a buried, hydrophobic microenvironment became exposed when WPI was succinylated, similar to behavior observed in Bence-Jones proteins (*37*) and heat-induced gelation of whey proteins (*38*). The data in **Figure 7** also agree with a previous study of succinylated whey proteins using steady-state fluores-

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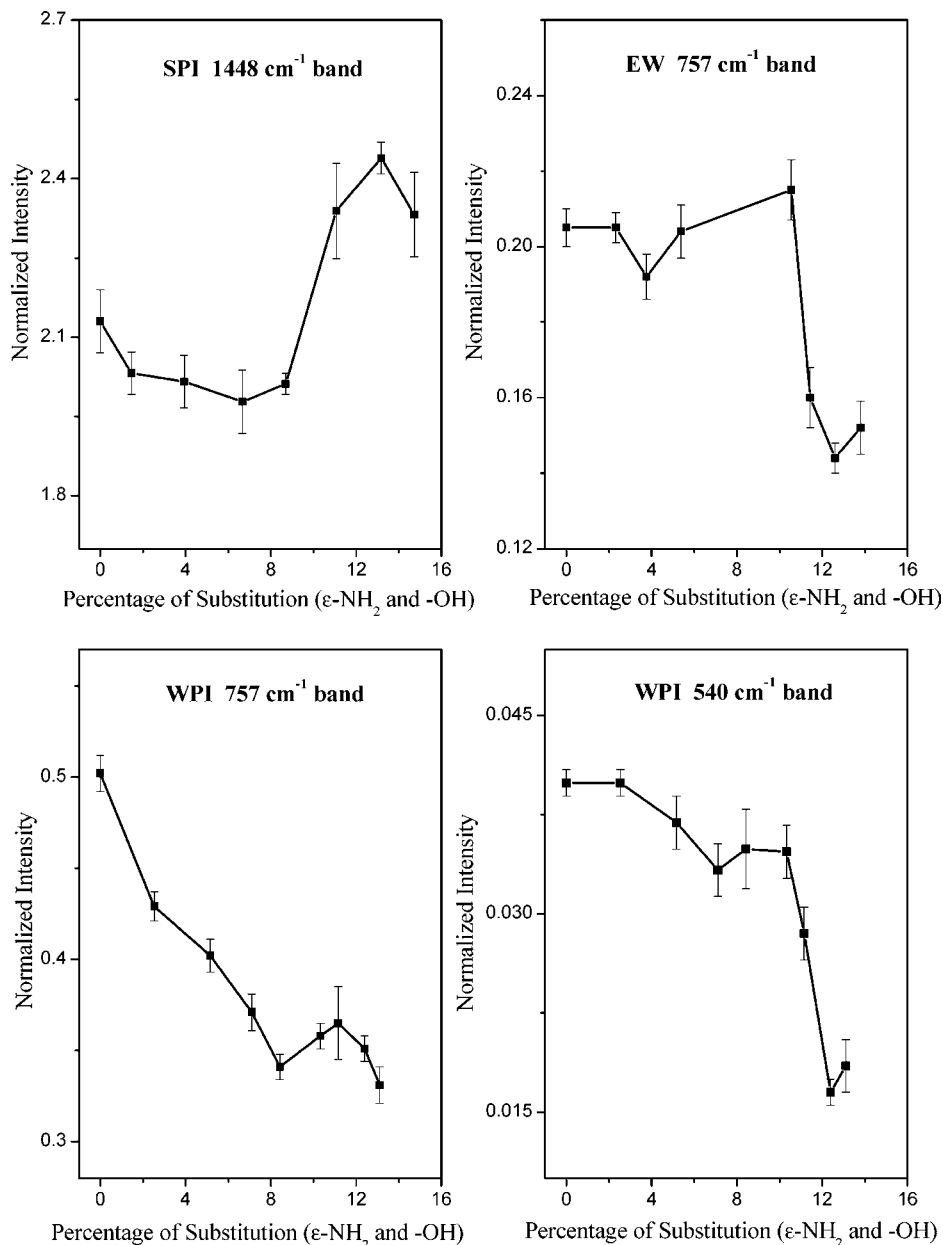*J. Agric. Food Chem.,* Vol. 52, No. 7, 2004 **1821**

Figure 7. Effect of succinylation on the normalized intensity of several Raman bands in SPI, EW, and WPI. The error bars represent standard deviations of duplicate determinations.

cence spectroscopy (*39*). Decreases in the tryptophan band intensity were also observed in succinylated EW, although the decrease was more pronounced at a later stage of succinylation (**Figure 7**). It was suggested that the attachment of the bulky succinate group, with all the associated negative charge, imposed a more flexible structure on the molecule (*39*).

Raman spectroscopy provides a direct analysis of the disulfide region. For SPI, a major band was observed at about 546 cm^{-1} (**Figure 2**), which has been assigned to the "trans−gauche−trans" conformation and can be attributed to the cystine residues (*23*, *37*). It has also been suggested that the C−H bending vibration of the aliphatic side chains of tryptophan also contributes to minor bands near 540 cm^{-1} (*23*, *40*). For WPI, a band appeared at 507 cm^{-1} (**Figure 4**) which has been assigned to the "gauche−gauche−gauche" conformation, indicating that some of the disulfide bonds in this protein are in the lower potential energy conformation. The intensity of the 540 cm^{-1} band decreased progressively with increase in the percentage

of substitution (**Figure 7**), whereas the 507 cm^{-1} band intensity was not changed markedly (data not shown). These data show either a conformational shift of the cystine residues or changes in trytophan microenvironment in WPI. Progressive decreases in disulfide band (around 508 cm^{-1}) intensity were observed during thermal gelation of whey proteins, suggesting protein unfolding (*37*). The Raman spectra of EW (control and modified) did not show sharp bands in the S−S region, and no marked changes in intensity were observed in the succinylated samples (**Figure 3**).

It is interesting to note that modification of the aliphatic hydroxyl groups (at a later stage of succinylation) seems to induce more pronounced conformational changes than the substitution of lysine residues (during the initial stage of succinylation). Other researchers (*25*, *41*, *42*) have shown that there are two principal stages of acylation with regard to conformational changes. In the first stage, *N*-acylation up to 60−70% does not lead to large changes in the conformation,

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whereas drastic changes occur in the second stage after passing through a "critical" threshold of acylation. This critical threshold of modification coincides with the appearance of a large amount of *O*-acylation, which contributes markedly to changes in charge and surface hydrophobicity.

In this investigation, we demonstrated the versatility of Raman spectroscopy in studying succinylated food proteins. It can be used as a simple, fast, and accurate analytical tool to determine the level of substitution of specific amino acid residues (lysine, serine, and threonine) in the modified proteins, thereby replacing the conventional wet chemistry methods, which are generally time-consuming and require handling of dangerous chemicals. From the same Raman spectra, valuable qualitative and quantitative data can also be obtained to monitor conformational changes during protein modification, which have been shown to be closely correlated to observed changes in physicochemical properties and improvement in functional performance, the main objective for succinylation of many food proteins. The data suggest that Raman spectroscopy could be used to measure levels of modification in other chemically modified food proteins, as long as changes in new or existing Raman bands can be quantitatively measured that are attributable to the modifications of specific amino acid residues or their functional groups.

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