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Raman spectroscopic determination of extent of O-esterification in acetylated soy protein isolates

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Abstract

Soy protein isolates were chemically modified with varying amounts of acetic anhydride, and the extent of O-esterification was determined by a standard wet chemistry method. Raman spectra of the modified soy proteins were obtained. A characteristic 1737 cm^{-1} C=O vibrational band was observed and its intensity was dependent on the degree of O-esterification. The ratio of intensity of the 1737 cm^{-1} band to a 1003 cm^{-1} phenylalanine stretch band (used as an internal standard) was plotted against the extent of O-esterification determined chemically. A linear fit was obtained with a correlation coefficient (R^2) of 0.9986. Hence, Raman spectroscopy represents a rapid non-destructive method to determine the degree of O-esterification in food proteins.
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Keywords: Raman spectroscopy; Acetylation; Soy protein isolates

1. Introduction

Soy protein isolates (SPI) are widely used by the food industry as functional ingredients in the manufacturing of many processed foods. In the food industry, chemical modification is often performed to expand the range of protein functional properties. Acetylation with acetic anhydrides has been shown to be a very powerful tool for improving the functional properties of SPI (Franzen & Kinsella, 1976a; Umeya, Mitsuishi, Yamauchi, & Shibasaki, 1981) and many plant proteins, including leaf protein (Franzen & Kinsella, 1976b), sunflower (Kabirrullah & Wills, 1982), cottonseed (Rahma & Narasinga Rao, 1983), winged bean (Narayana & Narasinga Rao, 1984), oat (Ma, 1984), faba bean (Muschiolik, Dickinson, Murray, & Stainsby, 1987), rapeseed (Gruener & Ismond, 1997), chickpea (Liu & Hung, 1998), and mung bean (Ei-Adaway, 2000). The related changes in functional and physicochemical properties of food proteins are affected by the degree of modification (Schwenke, 1997). Although the ε-amino group of lysine is the most readily acylated group in proteins, the acylating reagents can also react with other nucleophilic groups, such as phenolic (tyrosine) and aliphatic (serine and threonine) hydroxyl groups (Howell, 1996). The level of acylation (esterification) of hydroxy amino acids increases sharply when the reaction with the lysine is essentially complete (Fraenkel-Conrat, 1959; Gounaris & Perlamnn, 1967; Schwenke, Zirwer, Gast, Gornitz, Linow, & Gueguen, 1990). Furthermore, it has been documented that drastic conformational changes occurred with the appearance of a large amount of O-esterification, which contributes markedly to changes in charge and hydrophobicity (Schwenke, Mothes, Zirwer, Gueguen, & Subirade, 1993a; Schwenke, Dudek, mothes, Raab, & Seifert, 1993b). Therefore, it is important to measure the level of O-esterification to control quantitatively the desired degree of acetylation of a protein for use in different processes.

Traditionally, the extent of O-esterification in chemically modified proteins is measured using wet chemistry techniques (Habeeb & Atassi, 1969; Hall, Trinder, & Givens, 1973; Hestrin, 1949). These methods are

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destructive, involve time-consuming sample preparation procedures, and require relatively large amounts of samples. Furthermore, the wet chemistry methods are not amenable to continuous monitoring for quality control. Thus, it would be most convenient to select an analytical method that could use solid proteins without any need for sample preparation as well as to provide a direct, non-destructive, and faster determination of the degree of acetylation in the modified proteins.

Recently, Phillips, Xing, Liu, Chong, and Corke (1999a, 1999b) developed an analytical method using Raman spectroscopy for the determination of degree of acetylation and succinylation in modified starches. Raman spectroscopy can analyze samples directly, in 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 (Hendra, Jones, & Warnes, 1991; Wetzel & LeVin, 1999). Raman spectroscopy has been used as a quantitative analytical tool in the pharmaceutical and polymer industries (Hendra et al., 1991) and its potential in food analysis has been demonstrated (Davies, Binns, Melia, & Bourgeois, 1990; Ma & Phillips, 2002; Ozaki, Cho, Ikegaya, Muraishi, & Kawauchi, 1992; Sadeghi-Jorbachi, Wilson, & Belton, 1991; Thygesen, Lokke, Micklander, & Engelsen, 2003).

In this paper, we describe the development of an analytical technique based on Raman spectroscopy to determine the extent of O-esterification in acetylated SPI. Esterification of hydroxy amino acids by acetic anhydride appends ester carbonyl groups to the protein and these groups contain C=O bonds that exhibit a characteristic Raman vibration at around 1737 cm^{-1}. We will demonstrate that the intensity of this C=O vibrational band has a linear relationship with the extent of O-esterification of the soy protein, and the band is sufficiently intense to be conveniently used for quick and accurate determination of the extent of O-esterification in proteins. To our knowledge, this is the first reported use of Raman spectroscopy to determine the extent of O-esterification of chemically modified SPI.

2. Materials and methods

2.1. Acetylation

Commercial SPI, Supro 610, were obtained from Protein Technology International (St. Louis, MO). It was acetylated according to the procedure described by Franzen and Kinsella (1976a) with some modifications of the procedure. A dispersion (≈2.5%, w/v) of SPI was prepared by mixing 2 g of protein sample with approximately 80 ml distilled water followed by 1 h magnetic stirring. The pH of the dispersion was adjusted to about 8.0 by adding 2 N sodium hydroxide. Acetylation was accomplished by the addition of small aliquots (0.1 ml increments) of acetic anhydride to the protein dispersion. The quantities of acetic anhydride added were 0.1, 0.2, 0.4, and 0.8 g/g protein. The pH was maintained between 7.5 and 8.5 by the addition 2 N NaOH. After being stabilized at about 8.0, the pH was kept constant for another hour to allow the reaction to go to completion. The final pH was then decreased to 7.0 with 1 N HCl to prevent further modification. A control was prepared by the same procedure without the addition of acetic anhydride. The solutions were dialyzed at 4 °C for 48 h to remove impurities and excess reagents, and the proteins were recovered by freeze-drying.

2.2. Determination of extent of esterification of hydroxy amino acids

The quantity of acetylated hydroxy amino acids in modified SPI was measured by the alkaline hydroxylamine method as described by Gounaris and Perlamnn (1967). The reagent consisted of 2 M $NH_2OH \cdot HCl$, 3.5 M NaOH, and 0.05 M borate buffer in the proportions of 2:1:1, v/v/v. This reagent (2 ml) was added to 1 ml protein solution (5 mg/ml) and was incubated at 40 °C for 2 h. The reaction was stopped by the addition of 1.0 ml concentrated HCl (diluted 1:3, v/v, with water) followed by 1.0 ml 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 the addition of $FeCl_3$. The number of ester groups was obtained from a calibration curve using tyrosine ethyl ester as a standard.

2.3. Collection and analysis of Raman spectra

Raman spectra were recorded on a Renishaw-Raman Imaging Microscope (system 1000) equipped with a 514 nm Argon ion laser excitation (Spectra Physics) using about 100 mW of power. The laser was focused on the solid samples which were 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 collected in duplicate or triplicate, and the results were reported as the averages of these replicates.

3. Results and discussion

The extent of modification of the functional groups of a protein can be varied widely by changing the amount of the acylating agent employed. O-esterification occurs

at a sufficiently high excess of the reagent (Fraenkel-Conrat, 1959; Gounaris & Perlamnn, 1967; Schwenke et al., 1990). The Raman spectra of the control non-acetylated SPI sample and four acetylated SPI samples with different levels of modification are shown in Fig. 1. Assignment of some major Raman bands was made based on results from previous workers (Li-Chan, Nakai, & Hirotsuka, 1994; Peticolas, 1995; Tu, 1986). The intensity and location of the phenylalanine band at 1003 cm^{-1} band is not sensitive to conformation or micro-environment, and therefore can be used as an internal standard (Li-Chan, 1996). In comparison with the unmodified proteins, acetylated SPI exhibited a new peak at 1737 cm^{-1}, which provided a convenient marker band (Fig. 1A–E). Acetylation of hydroxy amino acids introduces additional ester carbonyl groups to the protein. Carbonyl compounds give rise to a strong band at 1900–1550 cm^{-1} caused by the stretching of the C=O bond, and carbonyl groups of ester are characterized by the absorption due to the C=O stretching frequency near 1740 cm^{-1} (Colthup, Daly, & Wiberley, 1990). The band at 1737 cm^{-1} can be attributed to the C=O bonds of ROOC– groups introduced by esterification. The progressive increases in peak intensity at 1737 cm^{-1} in acetylated SPI samples suggest that the intensity changes can be correlated with the extent of esterification of hydroxy amino acids in the protein.

Table 1 lists the ratio of the Raman intensity of the 1737 cm^{-1} C=O vibrational bands to 1003 cm^{-1} phenylalanine bands and the extent of esterification of hydroxy amino acids determined by the wet chemistry method for each of the soy protein samples. Fig. 2 shows a plot of the Raman band ratio against the extent of O-esterification. A linear regression line was plotted to the data ($y = mx + b$; where y is the ratio of the 1737 cm^{-1} band to 1003 cm^{-1} band, x is the extent of O-esterification, $m = 0.1853$, and $b = 0.0037$), with a correlation coefficient (R^2) of 0.9986. This conclusively demonstrates that the 1737 cm^{-1} Raman band intensity has a strong linear relationship with the extent of O-esterification for the modified SPI. We also noted that zero intensity for the Raman intensity is almost within the uncertainty of the linear regression y-intercept ($b = 0.0037$) and this indicates that the 1737 cm^{-1} peak is primarily due to esterification of the SPI samples with very little contribution from the parent SPI sample. Using

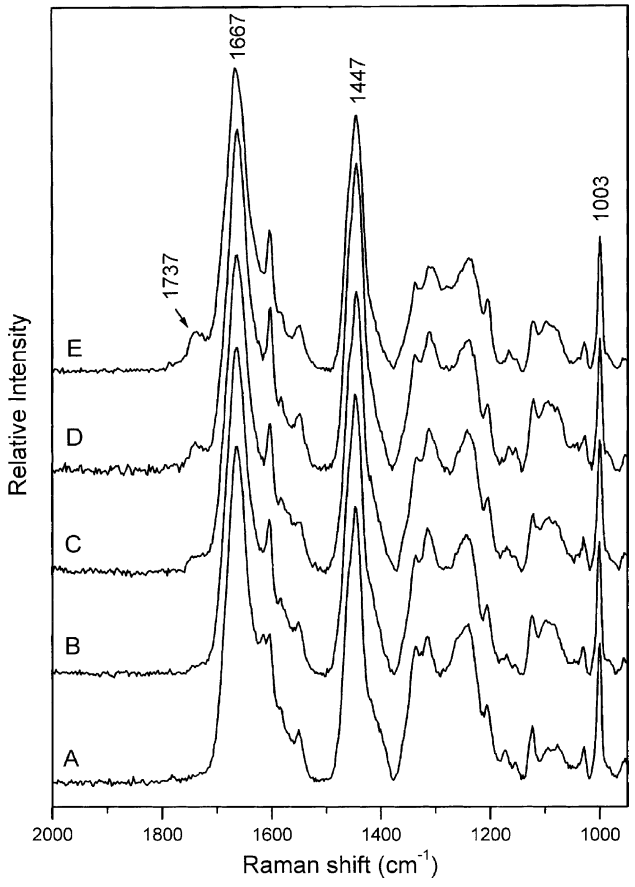

Fig. 1. Raman spectra of control non-acetylated soy protein isolates (A) and four acetylated soy protein isolates (B–E) with varying degree of O-esterification.

Table 1
Ratio of intensity of Raman 1737 cm^{-1} band to the 1003 cm^{-1} band for soy protein isolates vs. the extent of O-esterification

Soybean protein sample	Extent of O-esterification (mmol/g protein)[a]	I_{1737}/I_{1003}
Control (no acetylation) (A)	0.0000	0.005 ± 0.005
Sample 1 (B)	0.476 ± 0.002	0.093 ± 0.002
Sample 2 (C)	0.961 ± 0.002	0.176 ± 0.003
Sample 3 (D)	1.194 ± 0.005	0.223 ± 0.001
Sample 4 (E)	1.416 ± 0.004	0.271 ± 0.004

[a] Determined in triplicate by the hydroxylamine and $FeCl_3$ method (Gounaris & Perlamnn, 1967), with standard deviations indicated.

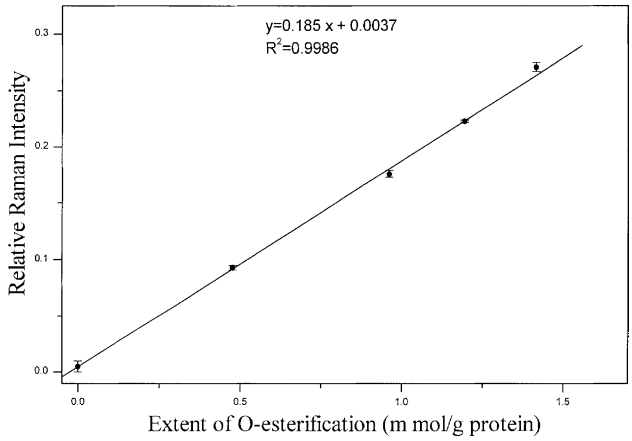

Fig. 2. Plot of the ratio of the Raman 1737 cm^{-1} C=O vibrational band to the 1003 phenylalanine stretching band vs. the degree of O-esterification determined by the hydroxylamine and $FeCl_3$ method for soy protein isolates. The line is a best-fit line to the plotted data. Error bars represent standard deviations of the means.

this calibration curve, we can obtain the extent of O-esterification and predict whether acetylation of the ε-amino groups of lysine is at a high level. Since the ε-amino groups of lysine are the most readily acetylated groups in proteins, their modification has a major impact on the physicochemical and functional properties of the final products (Howell, 1996). An estimate of the extent of acetylation of lysine is therefore important in controlling the functional performance of modified proteins.

4. Conclusions

The Raman spectroscopic method presented in this paper has several important advantages over other methods for the determination of level of esterification in modified proteins. The Raman method requires almost no sample preparation for the solid soy proteins which makes the method much more convenient as well as potentially faster. The technique is non-destructive whereas the wet chemistry methods are destructive tests usually requiring toxic and/or corrosive reagents. The Raman method also has the potential to be developed for routine quality control and fast analysis.

In conclusion, we have reported a plot of the 1737–1003 cm^{-1} Raman intensity ratios vs. the extent of O-esterification in acetylated SPI. This calibration curve shows a very high degree of linearity and would be suitable for determining the extents of O-esterification of soy protein isolates with unknown extents of O-esterification from their Raman spectra. Since only one batch of soy protein isolates has been tested, additional data would be required to determine the practical utility of this technique as a routine quality control procedure for acetylated soy isolates and other soy protein products (concentrates and flour, etc.). Further experiments are currently being conducted in our laboratory to assess the performance of the Raman spectroscopic technique for other chemically modified food proteins.

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References

Colthup, N. B., Daly, L. H., & Wiberley, S. E. (1990). *Introduction to infrared and Raman spectroscopy*. San Diego: Academic.

Davies, M. C., Binns, J. S., Melia, C. D., & Bourgeois, D. (1990). Fourier transform Raman spectroscopy of polymeric biomaterials and drug delivery systems. *Spectrochimica Acta A, 46*, 277–283.

Ei-Adaway, T. A. (2000). Functional properties and nutritional quality of acetylated and succinylated mung bean protein isolate. *Food Chemistry, 70*, 83–91.

Fraenkel-Conrat, H. (1959). Methods for investigating the essential groups for enzyme activity. *Methods in Enzymology, 4*, 247–269.

Franzen, K. L., & Kinsella, J. E. (1976a). Functional properties of succinylated and acetylated soy proteins. *Journal of Agricultural and Food Chemistry, 24*, 788–795.

Franzen, K. L., & Kinsella, J. E. (1976b). Functional properties of succinylated and acetylated leaf protein. *Journal of Agricultural and Food Chemistry, 24*, 914–919.

Gruener, L., & Ismond, M. A. H. (1997). Effect of acetylation and succinylation on functional properties of the canola 12S globulin. *Food Chemistry, 60*, 513–520.

Gounaris, A. D., & Perlamnn, G. E. (1967). Succinylation of pepsinogen. *The Journal of Biological Chemistry, 242*, 2739–2745.

Habeeb, A. F. S. A., & Atassi, M. Z. (1969). Enzymic and immunochemical properties of lysozyme-II. *Immunochemistry, 6*, 555–566.

Hall, R. J., Trinder, N., & Givens, D. I. (1973). Observations on the use of 2,4,6-trinitrobenzenesulphonic acid for the determination of available lysine in animal protein concentrates. *Analyst, 98*, 673–686.

Hendra, P. J., Jones, C. H., & Warnes, G. M. (1991). *Fourier transform Raman spectroscopy, instrumentation and chemical applications*. Chichester: Ellis Harwood.

Hestrin, S. (1949). Acylation reactions mediated by purified acetylcholine esterase. *The Journal of Biological Chemistry, 180*, 879–880.

Howell, N. K. (1996). Chemical and enzymatic modifications. In S. Nakai & H. W. Modler (Eds.), *Food proteins: properties and characterization* (pp. 260–268). New York: VCH Publishers.

Kabirrullah, M., & Wills, R. B. H. (1982). Functional properties of acetylated and succinylated sunflower protein isolate. *Journal of Food Technology, 17*, 235–236.

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

Li-Chan, E. C. Y., Nakai, S., & Hirotsuka, M. (1994). Raman spectroscopy as a probe of protein structure in food systems. In R. Y. Yada, R. Y. Jackson, & L. L. Smith (Eds.), *Protein structure–function relationship in foods* (pp. 163–197). London: Blackie Academic.

Liu, L. H., & Hung, T. V. (1998). Functional properties of acetylated chickpea proteins. *Journal of Food Science, 63*, 331–337.

Ma, C. Y. (1984). Functional properties of acylated oat protein. *Journal of Food Science, 48*, 1128–1131.

Ma, C. Y., & Phillips, D. L. (2002). FT-Raman spectroscopy and its applications in cereal science. *Cereal Chemistry, 79*, 171–177.

Muschiolik, G., Dickinson, E., Murray, E., & Stainsby, R. (1987). Interfacial and emulsifying behaviour of acetylated field bean protein isolate. *Food Hydrocolloids, 1*, 191–208.

Narayana, K., & Narasinga Rao, M. S. (1984). Effect of acetylation and succinylation on the functional properties of winged bean flour. *Journal of Food Science, 49*, 547–550.

Ozaki, Y., Cho, R., Ikegaya, K., Muraishi, S., & Kawauchi, K. (1992). Potential of near-infrared Fourier transform Raman spectroscopy in food analysis. *Applied Spectroscopy Reviews, 46*, 1503–1507.

Peticolas, W. L. (1995). Raman spectroscopy of DNA and proteins. *Methods in Enzymology, 226*, 389–416.

Phillips, D. L., Xing, J., Liu, H. J., Chong, C. K., & Corke, H. (1999a). Raman spectroscopic determination of the degree of succinate in modified waxy maize starches. *Analytical Letters, 32*, 2703–2711.

Phillips, D. L., Xing, J., Liu, H. J., Chong, C. K., & Corke, H. (1999b). General application of Raman spectroscopy for the determination of level of acetylation in modified starches. *Cereal Chemistry, 76*, 439–443.

Rahma, E. H., & Narasinga Rao, M. S. (1983). Effect of acetylation and succinylation of cottonseed flour on its functional properties. *Journal of Agriculture and Food Chemistry, 31*, 351–355.

Sadeghi-Jorbachi, H., Wilson, R. H., & Belton, P. S. (1991). Quantitative analysis of oils and fats by Fourier transform Raman spectroscopy. *Spectrochimica Acta A, 47*, 1449–1458.

Schwenke, K. D., Zirwer, D., Gast, K., Gornitz, E., Linow, K. J., & Gueguen, J. (1990). Changes of the oligomeric structure of legumin from pea (*Pisum sativum* L.) after succinylation. *European Journal of Biochemistry, 194*, 621–632.

Schwenke, K. D., Mothes, R., Zirwer, D., Gueguen, J., & Subirade, M. (1993a). Modification of the structure of 11S globulins from plant seeds by succinylation. In K. D. Schwenke & R. Mothes (Eds.), *Food proteins: structure and functionality* (pp. 143–187). Weinheim: VCH.

Schwenke, K. D., Dudek, St., mothes, R., Raab, B., & Seifert, A. (1993b). Physicochemical and enzymatic studies on acetylated protein isolates from faba bean. *Nahrung, 37*, 258–271.

Schwenke, K. D. (1997). Enzyme and chemical modification of protein. In S. Damodaran & A. Paraf (Eds.), *Food proteins and their applications* (pp. 394–414). New York: Marcel Dekker.

Thygesen, L. G., Lokke, M. M., Micklander, E., & Engelsen, S. B. (2003). Vibrational microspectroscopy of food. Raman vs. FT-IR. *Trends in Food Science Technology, 14*, 50–57.

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.

Umeya, J. I., Mitsuishi, N., Yamauchi, F., & Shibasaki, K. (1981). Effect of acetylation on hardening and softening properties of soybean protein-water suspending systems. *Agricultural and Biological Chemistry, 47*, 1577–1581.

Wetzel, D. L., & LeVin, S. M. (1999). Microspectroscopy-imaging molecular chemistry with infrared microscopy. *Science, 285*, 1224–1225.