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Effects of acetylation and succinylation on the physicochemical properties of the canola 12S globulin. Part I

L. Gruener & M. A. H. Ismond

Department of Food Science, University of Manitoba, Winnipeg, Canada R3T 2N2

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Chemical modification is a means of improving the functional properties of food proteins, which are directly related to the physicochemical characteristics. The objective of this study was to determine the effects of acetylation and succinylation on the physicochemical properties of canola proteins. Increasing acylation resulted in dissociation of the protein structure, as was evidenced by a decrease in molecular weight and enthalpy of denaturation. Acylation caused an overall decrease in isoelectric point, this effect being more pronounced with succinylation as compared to acetylation. Aromatic and aliphatic hydrophobicity increased significantly following acetylation. Aromatic hydrophobicity significantly decreased while aliphatic hydrophobicity showed an overall significant increase following succinylation. The amino acid profile remained unchanged with the exception of a slight decrease in lysine in the succinylated and acetylated proteins, and a decrease in proline in the succinylated proteins. These results indicate that acylation has a significant impact on the physicochemical properties, particularly molecular weight and net charge, of the canola 12S globulin. © 1997 Published by Elsevier Science Ltd

INTRODUCTION

Canola, the dominant oilseed crop grown in Canada today, is a valuable source of protein with a well-balanced amino acid composition and a high content of sulphur-containing amino acids (Ohlson & Anjou, 1979). However, its use in food products is limited due to inferior functionality as compared to other commercially available protein isolates, and the presence of undesirable antinutritional factors.

The functional properties of a protein in a food system are related to its physicochemical characteristics, including factors such as molecular weight, amino acid composition, net charge and surface hydrophobicity. Particularly important in determining functionality are surface hydrophobicity, molecular weight and conformation, charge density and molecular flexibility (Kim & Rhee, 1989). Therefore, in order to modify proteins with the objective of obtaining good functionality, knowledge of the physical characteristics of the protein is imperative.

Acylation through acetylation and succinylation causes an overall decrease in surface charge, dissociation of the spatial structure of the protein, followed by unfolding of polypeptide chains. This results in an increase in molecular flexibility, which Kim and Kinsella (1986) report is extremely important in determining the surface properties of proteins. Furthermore, modifications in net charge may be related to the protein solubility.

In this study, canola protein was isolated through a protein micellar mass (PMM) method, a noncovalent approach to protein isolation, and various physicochemical properties of the PMM and acylated concentrates were examined. Specifically, alterations in molecular weight, conformation, amino acid profile and isoelectric point were analysed.

MATERIALS AND METHODS

Protein extraction method

Canola protein was isolated by a modification of the PMM procedure, using methodology as outlined by Murray *et al.* (1981). Samples of defatted canola (*Brassicu napus*) meal were stirred in 0.3 M NaCl (1:10 ratio) for 4 h then centrifuged for 10 min at 3000 *g* (4°C)

using a Sorvall Refrigerated Centrifuge, Model RC-3 (DuPont Co., Wilmington, DE). The supernatant was filtered through Whatman No. 4 filter paper under vacuum. The supernatant was then concentrated using a 10^3 molecular weight cut-off Spiral Ultrafiltration cartridge in an Amicon UF unit (model RA 2000, Oakville, ON). The pressure was maintained at 20 p.s.i with the volume of the supernatant being reduced eight times. The concentrated protein solution was diluted by six times its volume with cold distilled water. The solution was then stored at 4°C for 16 h to allow the protein micelles to precipitate. The protein micelles were recovered by centrifugation at 3000 *g* for 30 min (4°C). The supernatant was discarded and the protein was freeze-dried for 48 h to obtain the PMM.

Acylation of protein

Succinylation was performed according to the method of Hoagland (1966) with some modification. PMM (2% w/v), was dispersed in 0.075 M phosphate buffer pH 8. Succinic anhydride (Anachemia) was added in small increments with constant stirring over a 1 h period at levels of 2, 10, 50 and 100% of the weight of the PMM in the slurry. During the reaction, the pH was maintained between 8 and 8.5 with 3.5 M NaOH. Suspensions were then dialyzed for 24 h against distilled water at 4°C to remove the excess anhydride. A control was prepared in the same manner but the addition of anhydride was omitted. Following dialysis the suspensions were freeze-dried to obtain the succinylated protein concentrates.

The method of Ponnampalam *et al.* (1990) was used with some modification for acetylation of the PMM. PMM (2% w/v) was dispersed in distilled water and adjusted to pH 8.5 with 1 M NaOH. Acetic anhydride (Sigma) was added slowly with constant stirring (1, 2.5, 5 and 20% of the weight of the PMM in the slurry) for 1 h. The pH was maintained between 8 and 8.5 by the addition of 1 M NaOH. A control was prepared in the same manner without the addition of acetic anhydride. The suspension was dialyzed for 24 h against distilled water at 4°C to remove excess reagent. The acetylated concentrate was recovered by lyophilization.

Determination of extent of chemical modification

The degree of acylation was estimated by determination of free amino groups according to Habeeb (1966) with modifications by Gueguen *et al.* (1990). A 1% (w/v) solution of PMM was prepared in 0.05 M NaCl, pH 9.2, containing 0.29% SDS. One ml of 0.05 M Na_2HPO_4 and 1 ml of 0.1% TNBS were added to 1 ml of protein solution. The solution was allowed to react at 60°C for 2 h; then 1 ml of 10% SDS was added followed by 0.5 ml of 1 M HCl. The absorbance of the solution was read at 335 nm against a blank prepared using identical conditions but omitting the protein.

Physicochemical characteristics

Molecular determination by gradient PAGE
The molecular weights of PMM and all acylated samples were estimated by nondissociating gradient PAGE according to the procedure of Robard *et al.* (1971).

Determination of isoelectric point
The isoelectric point of each protein sample was determined in duplicate according to the method of Winter and Anderson (1977).

Fluorescence spectra
The intrinsic emission fluorescence spectrum of each protein sample was recorded in duplicate using a Perkin–Elmer LS-5 Fluorescence Spectrophotometer (Coleman Instruments Division, Oak Brook, IL). Protein samples of 0.01% in 0.01 M phosphate buffer pH 8 were excited at 280 nm to determine the fluorescence emission spectrum.

Differential scanning calorimetry (DSC) analysis
The method of Arntfield and Murray (1981) with some modification was used for DSC analysis. Protein samples were dissolved in 0.1 M NaCl to make a slurry of 20% w/w solids. The thermal properties of PMM and all acylated proteins were measured in triplicate using a Dupont 990 Thermal Analyzer with a 910 Differential Scanning Calorimeter Cell Base (Westech Industrial Ltd., Missisauga, ON). The results were expressed both in terms of the maximum heat flow into the protein (thermal denaturation temperature, Td in °C) as well as the enthalpy of denaturation (ΔH value expressed in Joules/g of sample).

Surface hydrophobicity
The surface hydrophobicity of all protein samples in triplicate was determined using two fluorescent probes: 1-anilino-8-napthalenesulfonate (ANS) to estimate the aromatic hydrophobicity (Hayakawa & Nakai, 1985); and *cis*-parinaric acid (CPA) to estimate the aliphatic hydrophobicity (Kato & Nakai, 1980). Protein samples were serially diluted with 0.01 M phosphate buffer pH 8.0 to obtain a range of protein concentrations from 0.015 to 0.60 mg ml^{-1}. A 10 μl aliquot of ANS (8 mM in the same buffer) or CPA (3.6 mM) was added to 2 ml of each sample, and the relative fluorescence intensity was measured with a Perkin–Elmer LS-5 fluorescence spectrophotometer (Coleman Instruments Division, Oak Brook, IL) using a slit width of 0.5 nm and a fixed scale of 1.0. Wavelengths of excitation and emission were 390 and 470 nm for ANS plus 325 and 420 nm for CPA. The fluorescence intensity of each solution without the probe was subtracted from that with the probe to obtain the net fluorescence intensity (FI) at each protein concentration. The initial slope of a plot of fluorescence intensity as a function of protein

concentration was used as an index of protein surface hydrophobicity (S_0).

Amino acid analysis

The amino acid profile of the PMM and acylated proteins was determined by high performance liquid chromatography (HPLC) according to the procedure of Andrews and Baldar (1985). To account for the loss of tryptophan during acid hydrolysis, tryptophan levels in the proteins were determined separately according to the spectrophotometric method of Messineo and Musarra (1972).

Statistical analysis

The statistical analyses were performed using a SAS statistical analysis software program package. Significant differences among treatments were determined by Duncan's Multiple Range Test ($p \leqslant 0.05$).

RESULTS AND DISCUSSION

Extent of chemical modification

The addition of succinic anhydride acylated 3, 48, 53 and 61% respectively of the ϵ-amino groups (Table 1). These samples were then referred to as 3% S-PMM, 48% S-PMM, 53% S-PMM and 61% S-PMM. Acetic anhydride, added at various levels modified 16, 26, 42 and 62% of the ϵ-amino groups (Table 1). These samples were then referred to as 16% A-PMM, 26% A-PMM, 42% A-PMM and 62% A-PMM.

As in other studies, acetic anhydride was found to be a more effective acylating reagent than succinic anhydride (Ball & Winn, 1982; Ponnampalam *et al.*, 1990). As acetic anhydride is a liquid, it is mixed more readily into the reaction medium. To achieve the same level of succinylation as that of acetylation, it is necessary to employ more succinic anhydride and increase the reaction time to facilitate dissolution of the anhydride (Ponnampalam *et al.*, 1990).

Molecular weight determination

The PMM showed an intense band at 500 000 and a lighter band at 850 000 (Fig. 1). The band at 500 000 probably represented the 12S globulin whereas that at 850 000 was likely indicative of 15–17S components which are aggregates of the 12S protein. As the level of succinylation increased, the molecular weight of the bands decreased. This corroborates the findings of Kabirullah and Wills (1982), who reported that the major band of native sunflower proteins moved further into the gel with increasing degrees of acylation. This effect is likely due to dissociation of the subunits as a result of acylation. At a level of 53% succinylation, the 12S band was still evident, but a band at 50 000, which corresponds to the 2-3S subunit, also appeared. At the 62% level of succinylation, the 12S band disappeared and only the 2-3S band was present, indicating complete dissociation of the protein into subunits. This may be expected, as Schwenke *et al.* (1986) had previously characterized 60% modification to be the critical level for globulins from plant seeds. Schwenke *et al.* (1991) reported similar results with fababean protein, in which the 11S band showed increased mobility up to 60% modification, at which point the appearance of bands which moved more quickly indicated dissociation of the oligomeric protein components.

Table 1. Extent of succinylation and acetylation of canola 12S globulin as a function of ratio of succinic and acetic anhydride to protein respectively

Ratio of succinic anhydride to protein ($g\ g^{-1}$)	succinylation (%)	Ratio of acetic anhydride to protein ($g\ g^{-1}$)	acetylation (%)
0.02	3	0.01	16
0.1	48	0.025	26
0.5	53	0.05	42
1.0	61	0.20	62

The acetylated proteins exhibited a similar banding pattern, but the extent of dissociation was not as pronounced, as indicated by a lesser decrease in molecular weight as compared with succinylation. Acetylation does not cause as extensive dissociation as succinylation due to decreased charge effects. The 7S fraction was not apparent on the gel, which may be expected as Prakash & Rao (1986) have determined the 7S component to be transient in *Brassica* spp.

Isoelectric point determination

A single band was observed for PMM, which corresponds to an isoelectric point of 7.1 (Table 2). This is in agreement with the value of 7.2 reported in the

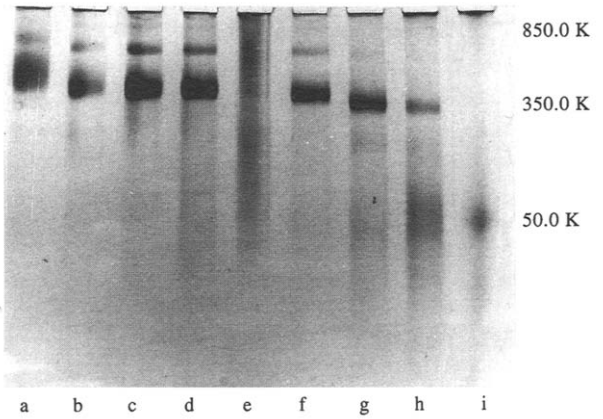

Fig. 1. Gradient PAGE electrophoregrams of PMM and all acylated proteins. a, PMM; b, 16% A-PMM; c, 26% A-PMM; d, 42% A-PMM; e, 62% A-PMM; f, 3% S-PMM; g, 48% S-PMM; h, 53% S-PMM; i, 61% S-PMM.

literature for the 12S canola globulin (Schwenke *et al.*, 1983). The succinylated proteins exhibited a successive decrease in the range of isoelectric points as the extent of acylation increased. The band with the highest intensity was found at pH 4.9 for the first three levels of succinylation. This increase in net negative charge is a result of the replacement of the positively charged amino groups with the carboxyl anions of the succinate half-amide (Canella *et al.*, 1979). At the highest level of succinylation (61% S-PMM), the most intense band corresponded to an isoelectric point of 5.2, which is slightly higher than the previous level of succinylation. Gueguen *et al.* (1990) reported a similar increase in isoelectric point with excessive succinylation, and the band was assumed to correspond to the modified subunits.

A similar pattern of decreasing isoelectric point with increasing level of acylation was noticed with the acetylated proteins. However, the decrease in isoelectric point was not as pronounced as those observed with succinylation, due to the acetyl group being neutral rather than anionic. As given in Table 2, multiple bands appeared for most acylated proteins; however, for each treatment there was a distinct major band with one or two accessory bands. It has been documented that multiple minor bands could result from an interaction of the protein with the constituent ampholytes. These multiple bands do not necessarily indicate sample denaturation or contamination (Pharmacia, 1982).

Intrinsic fluorescence spectra

The λ_{max} of the fluorescence emission spectrum of PMM was determined to be 340 nm (Table 2). As the degree of modification increased, a progressive increase in the λ_{max} (red shift) was observed. The maximum wavelength shift was an increase to 347 nm for the 61% S-PMM, and 346 nm for the 62% A-PMM. This indicates that the tryptophan residues in the protein became exposed to a more polar environment because of dissociation of the native oligomeric structure (Kim & Kinsella, 1986) and possible denaturation of the individual subunits. Lakkis and Villota (1992) observed similar shifts in fluorescence emission after acetylation of casein, BSA and whey protein.

Differential scanning calorimetry (DSC) analysis

The ΔH measures the enthalpy required for thermal denaturation of a protein. In this study, PMM was found to have a ΔH of 11.0 J g^{-1} (Table 2). The ΔH values decreased significantly at each level of succinylation. At the highest level of succinylation the endotherm completely disappeared, indicating complete denaturation or unfolding of the subunits. For the acetylated proteins, a significant decrease in ΔH values was also observed, but this effect was not as dramatic as those of the succinylated proteins. At the highest level of acetylation, the endotherm also disappeared. This supports the theory of Schwenke *et al.* (1991) that 60% modification is the critical level for denaturation of plant proteins.

The temperature of denaturation (*Td*) denotes the temperature required to denature a protein. The PMM had a *Td* of 87.6°C (Table 2), and this value decreased significantly with each increasing level of succinylation, indicating unfolding of the protein structure. The first

Table 2. Temperature of denaturation, enthalpy of denaturation, surface hydrophobicity, maximum λ of fluorescence emission, and isoelectric point of acylated concentrates[1]

Treatment	*Td* (°C)[2]	ΔH(J g^{-1})[2]	S_0 (aromatic)[2]	S_0 (aliphatic)[2]	λ_{max}[3]	*pI*
PMM	87.6 ± 0.2^{b}	10.97 ± 0.52^{a}	97.5 ± 1.8^{bc}	222.4 ± 16.1^{cd}	340	7.1
3% S-PMM	87.1 ± 0.3^{c}	9.78 ± 0.78^{b}	85.1 ± 1.7^{d}	170.2 ± 8.2^{e}	340	5.5 5.3 4.9
48% S-PMM	84.2 ± 0.4^{d}	2.41 ± 0.61^{e}	55.5 ± 3.4^{f}	214.2 ± 17.4^{cd}	341	5.5 4.9 4.6
53% S-PMM	83.6 ± 0.2^{e}	1.06 ± 0.13^{f}	62.3 ± 1.5^{e}	238.3 ± 18.1^{c}	344	4.9 4.6
61% S-PMM	—	—	58.1 ± 0.8^{ef}	283.9 ± 6.4^{b}	348	5.2
16% A-PMM	89.0 ± 0.2^{a}	8.01 ± 0.39^{c}	93.0 ± 0.5^{c}	205.5 ± 7.6^{d}	340	6.1 5.5
26% A-PMM	89.0 ± 0.2^{a}	8.45 ± 0.41^{c}	84.4 ± 0.9^{d}	175.1 ± 2.3^{e}	340	5.7 5.4 5.1
42% A-PMM	88.8 ± 0.2^{a}	5.50 ± 0.49^{d}	101.1 ± 4.9^{b}	207.2 ± 7.0^{d}	341	5.3 5.1 5.0
62% A-PMM	—	—	322.6 ± 2.9^{a}	417.1 ± 19.7^{a}	346	5.8 4.9

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents a mean of three determinations.
[3] Each value represents a mean of two determinations.

three levels of acetylation exhibited a significant increase in *Td* from the PMM.

Surface hydrophobicity

The surface hydrophobicity of a protein is an index of the number of hydrophobic groups on the surface of a protein in contact with the polar aqueous environment. Surface properties of a protein are significant, as they are indicative of its capacity for intermolecular interaction, thereby influencing its functionality. According to Hayakawa and Nakai (1985), protein hydrophobicity may be classified into two types, aromatic and aliphatic. Several researchers have correlated surface hydrophobicity with functional properties. Li-Chan *et al.* (1985) reported that both aromatic and aliphatic hydrophobicity were significant predictors of emulsifying and fat-binding properties of salt soluble muscle proteins. However, a study by Hayakawa and Nakai (1985) on the thermal properties of ovalbumin found no difference between the two types of hydrophobicity measurements. Further research by Nakai and Li-Chan (1988) indicated that aromatic hydrophobicity showed a significant correlation with protein insolubility.

The aromatic hydrophobicity of PMM was 97.5 (Table 2). As the level of succinylation increased, the aromatic hydrophobicity showed a significant decrease, to a value of 58.1 at the highest level of modification. This corroborates the research of Paulson and Tung (1987) on canola proteins. In their study, a decrease in aromatic hydrophobicity of canola protein isolates was noticed with an increase of succinylation. Paulson and Tung (1987) suggested that as there is a higher frequency of charged groups resulting from succinylation which are assumed to be on the surface of the protein, it is likely to result in a lower frequency of nonpolar groups on the surface. This is supported by their report that, with increasing levels of succinylation, charge frequency and electronegativity increased while the surface hydrophobicity decreased. For the acetylated proteins, the aromatic hydrophobicity initially decreased before exhibiting a significant increase with the 62% A-PMM. Kim and Rhee (1989) observed a similar pattern with the aromatic hydrophobicity of acetylated soy proteins. This increase at high levels of acetylation could be due to dissociation of the subunits as positive charges are replaced with neutral charges. This resulted in a repulsion among subunits which would have made previously buried hydrophobic groups accessible to binding by ANS.

The aliphatic hydrophobicity of PMM was 222.4 (Table 2). The aliphatic hydrophobicity of the succinylated proteins initially decreased, then exhibited a significant increase at the highest level of modification. This is in accordance with the results of Ma *et al.* (1986) on the surface hydrophobicity of wheat gluten. In this study, the aliphatic hydrophobicity of the acetylated proteins demonstrated a similar pattern as with their aromatic hydrophobicity by decreasing initially then increasing.

Amino acid analysis

A slight decrease in lysine was noticed with succinylation and acetylation of the proteins, and a decrease in proline occurred following succinylation (Table 3). There was no significant difference in levels of tryptophan following acylation (data not shown). Several other researchers have investigated the effects of acylation on the amino acid content of proteins. Franzen and Kinsella (1976*a*) and Kabirullah and Wills (1982) reported little change in amino acid profile following acylation of soy proteins and sunflower proteins respectively. In addition, Franzen and Kinsella (1976*b*)

Table 3. Amino acid analysis of PMM and acylated concentrates[1]

	PMM	3% S-PMM	48% S-PMM	53% S-PMM	61% S-PMM	16% A-PMM	26% A-PMM	42% A-PMM	62% A-PMM
ASPARTIC ACID	8.48	9.13	9.16	9.26	8.64	9.81	9.81	9.71	9.47
THREONINE	3.43	3.69	3.48	3.48	3.53	3.83	3.83	3.76	3.70
SERINE	5.04	5.42	5.29	5.35	5.20	5.61	5.61	5.54	5.56
GLUTAMIC ACID	18.99	18.05	20.10	20.21	19.98	18.84	18.84	18.91	18.82
PROLINE	8.29	8.62	8.13	8.29	7.83	6.28	6.28	6.56	7.63
GLYCINE	6.01	6.07	6.34	6.29	6.21	6.07	6.07	6.07	6.21
ALANINE	4.24	4.64	4.52	4.50	4.41	4.87	4.87	4.95	4.79
CYSTEINE	1.78	1.51	1.49	1.49	1.74	1.19	1.19	1.27	1.39
VALINE	3.87	3.96	4.09	4.09	4.76	4.30	4.30	4.18	3.75
METHIONINE	1.76	2.33	2.26	2.20	1.64	2.25	2.25	2.27	2.27
ISOLEUCINE	3.42	3.24	2.96	2.88	3.59	3.72	3.72	3.65	3.19
LEUCINE	8.18	8.05	8.10	8.03	8.20	8.10	8.10	8.12	8.14
TYROSINE	2.91	3.07	2.97	3.01	2.81	3.00	3.00	2.97	3.34
PHENYLALANINE	5.22	5.33	5.15	5.15	5.10	5.21	5.21	5.16	5.18
HISTIDINE	3.27	2.59	2.58	2.59	2.65	2.71	2.71	2.74	2.31
LYSINE	4.66	3.77	2.70	2.47	2.94	3.92	3.92	3.92	4.06
ARGININE	8.31	8.33	8.31	8.37	8.44	7.74	7.74	7.75	8.04

[1]Expressed as percentage of total amino acids in proteins.

found that there was no apparent destruction of amino acids following acylation of leaf proteins. Ma and Wood (1987) noticed a slight decrease in phenylalanine in succinylated oat proteins, and a decrease in valine, leucine, isoleucine, phenylalanine and cystine in acetylated oat proteins.

Franzen and Kinsella (1976*a*) suggested that the effect of acylation on the nutritional value of proteins may be overcome by reducing the extent of acylation, as well as by supplementing the diet with lysine. Furthermore, acylated proteins which are intended for use as functional ingredients do not provide a substantial source of nutritive protein in the foods into which they are incorporated (Franzen & Kinsella, 1976*a*).

CONCLUSION

Overall, the acylated concentrates were found to decrease in molecular weight with increasing modification, indicating dissociation and possible denaturation of the protein structure. In addition to a decrease in molecular weight, the acylated concentrates exhibited isoelectric points that were lower than the value of 7.1 previously determined for the PMM. Changes in protein conformation caused by denaturation were also illustrated by a decrease in the enthalpy of the protein as the level of modified increased, as well as alterations in the fluorescence spectra. Determination of the surface hydrophobicity indicated an initial decrease followed by an increase in aromatic and aliphatic hydrophobicity of both acetylated and succinylated proteins with increasing levels of modification proteins. The amino acid profile remained unchanged with the exception of a slight decrease in lysine in the succinylated and acetylated proteins, and a decrease in proline in the succinylated concentrates. In general, the physicochemical characteristics of the modified proteins suggest there is a dissociation of the protein subunits with mild modification (3% succinylation, 16% acetylation) followed by denaturation of the subunits with extensive modification (61% succinylation and 62% acetylation).

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