Effect of Acylation on Substructural Properties of Proteins: A Study Using Fluorescence and Circular Dichroism

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Effect of succinylation, acetylation, and reductive alkylation on substructural properties of three milk protein systems (casein, BSA, and whey proteins) was studied. Three levels of modifications were achieved in each case, and changes in the proteins' spectral properties were determined. Casein attained the highest degree of modification for all of the treatments used. Acylation enhanced denaturation and improved surface hydrophobicity of all of the proteins. Modification of BSA resulted in a red-shifted emission fluorescence peaks; however, its circular dichroic (CD) patterns showed insignificant difference from those of the native. This may imply that acylation has affected only the tertiary structure of BSA. Fluorescence and CD profiles of whey proteins and caseins showed considerable changes in their conformational makeup. It appears that acylation may have a more drastic effect on multicomponent proteins, possibly by means of perturbing their protein–protein mode of interaction.

INTRODUCTION

Chemical modification of proteins has been used extensively in structure–function relationship studies to probe the reactivity of specific functional groups on a peptide chain (Habeeb et al., 1958; Means and Feeney, 1971; Qasim and Salahuddin, 1978). In the area of food proteins, and despite the fact that this practice is still in its infancy, a wealth of literature is currently available on the beneficial contribution of chemical modification to the functionality of various proteins (Kim and Kinsella, 1986; Ma and Holme, 1982; Montejano et al., 1984). Derivatization of food proteins by means of acylation has recently attracted considerable attention as an adequate alternative to alkalinization and acidification treatments and has been recommended to serve several purposes. Examples include blocking the reactivity of specific groups involved in some deteriorative reactions such as nonenzymatic browning (Bjarnson and Carpenter, 1969; Lakkis and Villota, 1992), improving surface properties (Franzen and Kinsella, 1976; Guegen et al., 1990; Rauschal et al., 1981), imparting thermal stability (Ball and Winn, 1982; Kitabatake et al., 1985), and facilitating separation, processing, and refining of plant proteins (Shetty and Kinsella, 1982) and in enhancing digestibility and nutritional value (Voustinas and Nakai, 1979). Acylation of the free amino groups on a polypeptide chain involves the utilization of reagents that react covalently at the amino sites of the proteins. The mechanism, depending on the nature of the modifying agent, can affect the charge balance of the molecule in three different ways, namely, by preserving the positive charge on the amino groups, by abolishing charge and bringing it to neutrality, or by imposing a negative charge as a substitute for the original positive charge.

Modification of the first class (i.e., preserved positive charge) includes reactions of the protein with reagents such as *N*-carboxy anhydrides (NCA) and succinimides, imido esters, acrylonitriles, aldehydes, and ketones (in the presence of sodium borohydride). NCA has been used as a vehicle to covalently introduce phenylalanine, tyrosine, methionine, and other amino acids to proteins (Li-Chan et al., 1979; Sen et al., 1981). Depending on the distribution and length of amino acid copolymers covalently linked to proteins, variations in the functional properties have often been reported (Li-Chan et al., 1979; Puigserver et al., 1979).

Acylation by means of charge abolishing is represented by acetylation. Choi et al. (1981) acetylated cottonseed protein and found an improvement in its hydration surfactancy properties. Minor improvements in solubility and foaming ability of acetylated leaf proteins were reported by Franzen and Kinsella (1976). Acetylated fish protein resulted in lower isoelectric point but depressed susceptibility to tryptic hydrolysis (Groninger, 1973; Groninger and Miller, 1975). Acylation by means of negative charge addition includes reactions of proteins with anhydrides of succinates, methyl succinates, glutarates, malates, and others. Compared to other acylation processes of this class, succinylation of food proteins was found to result in the most desirable functional qualities (Schwenke and Rauschal, 1980; Shetty and Kinsella, 1982). Damodaran and Kinsella (1981) showed that succinylation of soy proteins led to a substantial increase in their hydrophobicity accompanied by a tendency toward dissociation. Melnychym and Stapely (1973), in a patented study, suggested a process for succinylating vegetable proteins intended as ingredients for coffee whiteners. Compared to unmodified proteins, the succinylated ones showed desirable taste and agreeable texture. In addition, the product did not show any tendency toward feathering (precipitation mechanism of proteins upon interaction of the free amino groups with caffeic, chlorogenic, and tannic acids). Geugen et al. (1990) studied the effect of succinylation on the adsorption kinetics of solutions of 12S globulins and reported remarkable changes in surface tension of the protein. Despite the information available on the various effects of acylation on the functionality of food proteins, the contribution of the physicochemical nature of the acylating group per se or the indirect conformational changes in the proteins to the outcomes of these modifications remains a matter of controversy.

In view of our continued interest in studying the effect of modification on the substructural properties of milk proteins, this work has attempted to correlate structural changes taking place when the ϵ-amino sites of lysine residues are blocked to the nature of the acylating group. To establish a more generalized picture of this approach, three different protein systems were tested, namely casein, bovine serum albumin, and whey proteins. Structurally, the chosen proteins represented different classes of mo-

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Table I. Extent of Succinylation, Acetylation, and Alkylation of BSA, Casein, and WPI As Determined According to the TNBS Method[a]

protein	% modification								
	succinylation			acetylation			alkylation		
BSA	35.0	59.5	87.3	30.0	56.3	82.4	18.8	43.9	71.4
	(2.21)[b]	(6.71)	(3.72)	(1.01)	(2.24)	(3.00)	(1.51)	(4.91)	(6.32)
casein	40.8	67.1	92.0	37.0	56.6	87.3	35.0	45.6	79.0
	(1.11)	(3.00)	(1.98)	(3.11)	(5.20)	(5.15)	(4.20)	(3.39)	(4.53)
WPI	38.1	64.4	91.3	32.2	55.0	84.1	24.5	43.6	72.2
	(4.01)	(1.89)	(5.11)	(4.02)	(2.64)	(4.82)	(1.31)	(1.55)	(8.10)

[a] Percent modification of the lysine groups was calculated by assuming 0% change in the native proteins. Three levels of modification were attained for each protein by varying the molar concentration of the acylating reagent (see text). [b] Numbers in parentheses represent standard deviation.

lecular organization: casein, an unordered highly flexible protein; bovine serum albumin, a compact but flexible protein with high content of disulfide bonds; and whey proteins, a multicomponent system with highly compact and less flexible structure. To further investigate the contribution of steric hindrance and hydrophobic interaction to the structural conformation of the proteins, three types of acylation approaches were employed, i.e., succinylation, acetylation, and reductive alkylation. Three levels of modifications were attained in each case.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA, fraction V) was obtained from Sigma Chemical Co. (St. Louis, MO). Casein was purchased from ICN Biochemicals (Cleveland, OH), and whey protein isolates (WPI) were purchased from Le Sueur BioPro Isolates (Le Sueur, MN). Acetone was from Fisher Scientific (Fair Lawn, NJ). Succinic and acetic anhydrides, trinitrobenzenesulfonic acid (TNBS), and the rest of the chemicals were of analytical grade and were obtained from Sigma.

Methods. Succinylation of the three proteins was achieved using succinic anhydride at pH 8.5 (0.075 M phosphate buffer) according to the method of Franzen and Kinsella (1976). Acetylated proteins were prepared following the procedure of Hoagland (1966) using acetic anhydride in a saturated sodium acetate medium. Alkylation by means of methylation was done using acetone in borate buffer (pH 9.0) according to the general method of Means and Feeney (1968) as modified by Lee et al. (1978). Reductive methylation reaction was initiated by addition of sodium borohydride. Three levels of modifications were achieved in every case by varying the molar ratio of the acylating reagent to the number of free amino groups in the protein (110, 316, and 504).

The extent of modification of the free amino groups of native and modified proteins was determined using trinitrobenzenesulfonic acid (TNBS) according to the method of Fields (1970). The degree of acylation was expressed as percent change in the amino groups assuming 0% modification for the native/unmodified proteins.

Hydrophobicity of native and modified proteins was determined using the fluorescent probe, 1-anilino-8-naphthalenesulfonic acid (ANS) as described by Hayakawa and Kato (1985). Two-milliliter protein solutions of five various concentrations (0.008–0.03%) in phosphate buffer (pH 7.0) were prepared. Ten microliters of ANS (8.0 mM) in 0.1 M phosphate buffer was added to each protein concentration, and fluorescence intensity was recorded (wavelengths were 390 and 470 nm for excitation and emission, respectively). The initial slope of the plot of fluorescence intensity vs protein concentration was used to denote the hydrophobicity index of the protein.

Steady-state fluorescence measurements were carried out with an I.S.S. photon counter (I.S.S., Champaign, IL). Protein solutions (0.2 mg/mL) in pH 7.01 phosphate buffer containing 0.1 M KCl were placed in 1 cm path length quartz cuvettes (at 20 °C). Samples were excited at 295 nm (excitation wavelength of tryptophan), and their emission between 300 and 400 nm was scanned. Slit widths were 8 nm for excitation and 16 nm for emission.

Circular dichroic measurements were obtained using a Jobin-Ivon spectropolarimeter (Longjumeau, France) attached to an AT&T computer unit. The instrument was calibrated with 1-camphorsulfonic acid (CSA). A fused quartz cell (free of birefringence) of 0.1 cm path length was used for protein concentrations of 0.2 mg/mL in 0.1 M phosphate buffer (pH 7.01). The spectropolarimeter unit was programmed with a multiple scan and baseline subtraction capabilities. The scan rate was 2 nm/min, and the time constant was 2 s (at 20 °C). Each spectrum represented the average of at least five determinations of three separate scans. Data were expressed in terms of ellipticity, θ (mdeg). The unit was equipped with a digitizing accessory which allows smoothing of the CD curves by reducing noise and improving data precision. The content of α-helices, β-structures, and random coils of the native and modified proteins was estimated by comparing their spectra to those of a model polyamino acid; polylysine was chosen for this study due to its pronounced conformational changes in different media.

RESULTS AND DISCUSSION

Degree of Modification. Data on the degree of acylation of bovine serum albumin (BSA), casein, and whey protein isolates (WPI) are presented in Table I. As shown from these results, complete blockage of the ϵ-amino groups of lysine could not be attained by any of the proteins for any of the treatments employed.

A general feature of this experiment is the higher degree of succinylation of the three proteins as compared to their acetylated and alkylated counterparts. One explanation for this result could be the high affinity of the succinates (dicarboxylates) to the ϵ-amino groups of lysine as compared to that of the monocarboxylic acetates (Habeeb et al., 1958). Another factor may be related to the physicochemical nature of the acylating groups. Succinates, by virtue of their bulkiness and high net charge (−2/succinyl group), may affect the conformational structure of the polypeptide chain, thus forcing it to unfold and to expose the lysine groups inaccessible in the native protein. This mechanism is more important in globular proteins.

Another feature of these data is the ease of acylation of casein as reflected in a significantly higher degree of modification as compared to BSA and WPI that are characterized by a greater number of amino groups than caseins. However, casein, a highly flexible structure with high content of random coils, has most of its lysine residues at peripheral sites of the molecule. This aspect is, therefore, expected to allow easier contact with the acylating groups. Degree of alkylation of the three proteins was fairly low at all levels of reagent added.

Schwenke et al. (1986) noticed that incomplete blocking of the ϵ-amino groups upon acylation seems to be a general feature in 12S globulins from plant seeds. They correlated this observation to some structural peculiarity of these globulins, i.e., spatial structure and distribution of reactive amino groups resulting in steric hindrance and a possible

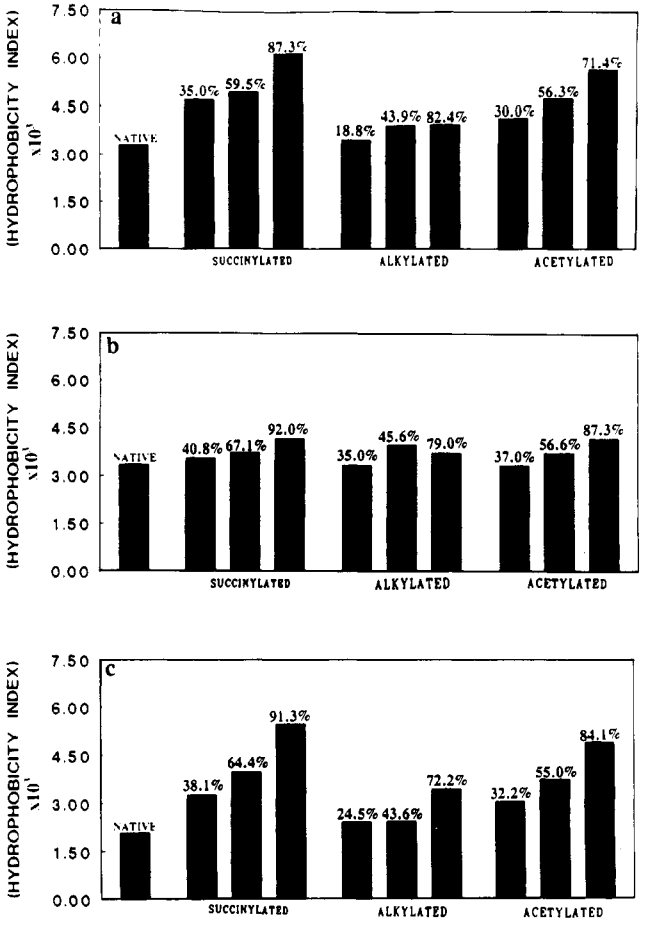

Figure 1. Hydrophobicity indices of native and modified proteins: bovine serum albumin (a), casein (b), and whey protein isolates (c), hydrophobicity was measured according to the method of Hayakawa and Kato (1985) using ANS as a fluorescent probe.

restraint to nucleophilic attack. Contradictory results, however, were found by Kim and Kinsella (1986) and Rao and Rao (1979), who prepared virtually completely acylated soy glycinin. Qasim and Salahuddin (1978) and, more recently, Batra et al. (1990) showed that complete blockage of the ϵ-amino groups of lysine residues was possible only in the presence of a high molar excess of acetic and succinic anhydrides.

Hydrophobicity. Surface hydrophobicity indices (measured by ANS fluorescent probe) of native and chemically modified proteins are presented in Figure 1. As evident from these data, surface hydrophobicity of the three proteins increased progressively in parallel with the degree of modification, indicating a change in the conformational structure of the proteins and a subsequent exposure of their hydrophobic cores to the ANS probe. Succinylated BSA, casein, and WPI showed more pronounced effect on the hydrophobicity of the proteins as compared to their acetylated and alkylated counterparts. This result is in good agreement with data on the degree of modification of these proteins. Acetylation resulted in a more pronounced effect on the hydrophobicities of WPI and BSA as compared to that of casein. Although acylation by means of reductive alkylation involves a reduction in the hydrophilicity of proteins by incorporating hydrophobic groups onto these sites, treatment of the three proteins under study resulted in a lower hydrophobicity than expected. Factors such as the low degree of alkylation as compared to acetylation and succinylation and the subsequent restricted structural relief may account for this feature. Another reason may be the higher sensitivity of the ANS probe toward aromatic hydrophobic sites over those of aliphatic ones, as is the case with the other commonly used probes such as *cis*-parinaric acid (Hayakawa and Nakai, 1985). Thus, the measured hydrophobicity is a good representation of the degree of denaturation and chain unfolding that have taken place upon acylation.

Alkylation of WPI showed enhanced hydrophobicity with increased degree of modification. Inconsistent trends were obtained for alkylated BSA or caseins. The latter protein has, in fact, shown a reversed order; i.e., at higher degrees of modification a slight drop in hydrophobicity index was obtained.

Investigations on the changes in hydrophobicity of other proteins upon acylation have resulted in a similar trend. Akita and Nakai (1990) showed that the structural unfolding of β-lactoglobulin upon incorporation of fatty acids was counteracted by a reverse mechanism at higher degrees of modification where hydrophobic interactions promoted polymerization and led to a sharp decrease in the ANS hydrophobicity. Haque and Kito (1983) studied the effect of palmitoyl lipophilization on the structural properties of casein and found a progressive increase in its surface hydrophobicity as more ligands were introduced into the protein. However, this trend was reversed when excess molar ratios of palmitoyl groups were added due to possible molecular associations and subsequent polymerization mechanisms.

Steady-State Fluorescence Profiles. Tryptophan emission spectra of native and modified proteins are shown in Figures 2–4. A notable feature of these spectra is the decrease in fluorescence intensity and the shifts in emission peaks toward longer wavelengths upon acylation. These trends are indicative of enhanced exposure of the indole chromophore of the proteins to the aqueous environment accompanied by some conformational changes and molecular unfolding.

As evidenced in Figures 2–4, spectra of succinylated BSA, casein, and WPI, respectively, were found to undergo more drastic shifts than those of their acetylated or alkylated counterparts. In general, the red-shifted fluorescence profiles followed a progressive pattern with increased degrees of modification and subsequent enhancement of the proteins' surface hydrophobicities. Succinylated WPI and BSA resulted in fluorescence peak shifts of 17 and 11 nm, respectively, at their highest degrees of modification, while casein showed a shift of only 8 nm. Despite the fact that casein attained higher degrees of modification than those of BSA and WPI (at all levels of molar concentration of succinic anhydride used), its spectral shifts were smaller. Many factors may have contributed to this result; of practical importance is the inherent molecular flexibility of casein and lower content of tertiary structure which are expected to lead to lower degree of molecular expansion upon acylation. On the contrary, the introduction of the bulky, negatively charged succinyl groups to the surface lysine residues of the compact-structured proteins (BSA and WPI) may have caused some expansion and/or fragmentation of the molecule resulting in the exposure of more tryptophan residues to the polar environment and thus to longer red shifts.

Kim and Kinsella (1986) studied the conformational changes of glycinin upon succinylation by monitoring three different parameters, namely relative viscosity and UV and fluorescence spectra. Their data showed that at high degrees of modification (above 75%) the relative viscosity of the protein increased sharply due to an increase in the hydrodynamic volume of the glycinin molecule. Qasim

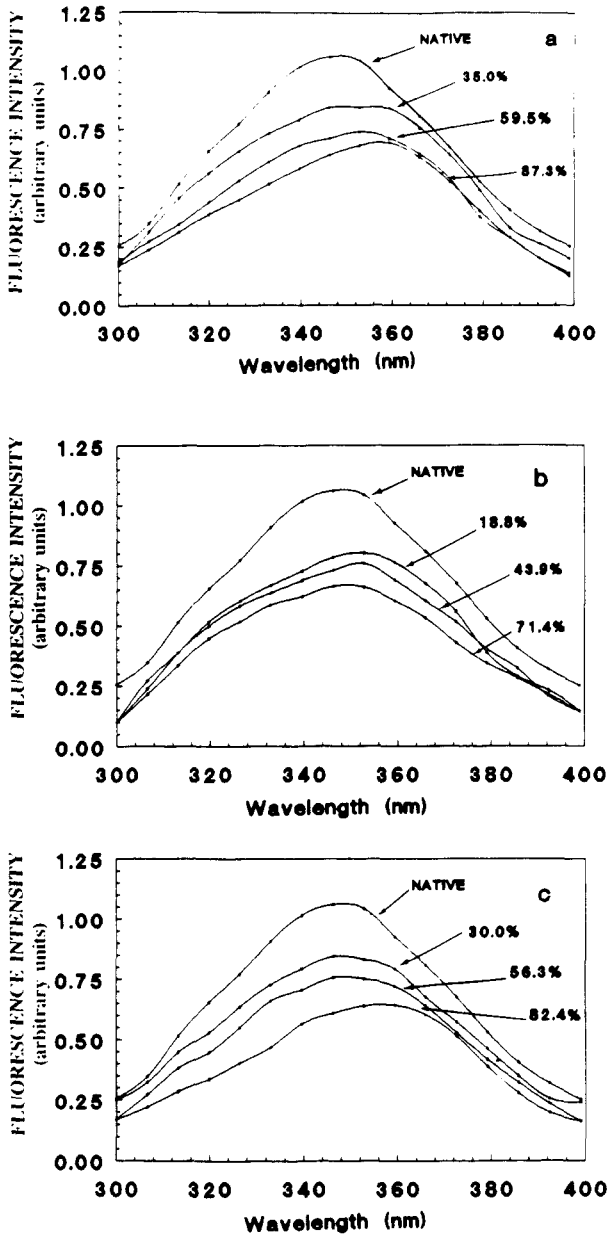

Figure 2. Fluorescence emission spectra of native and modified BSA: succinylated (a), alkylated (b), and acetylated (c). Samples of 0.02% concentration in 0.1 M phosphate buffer, pH 7.01, were excited at 295 nm.

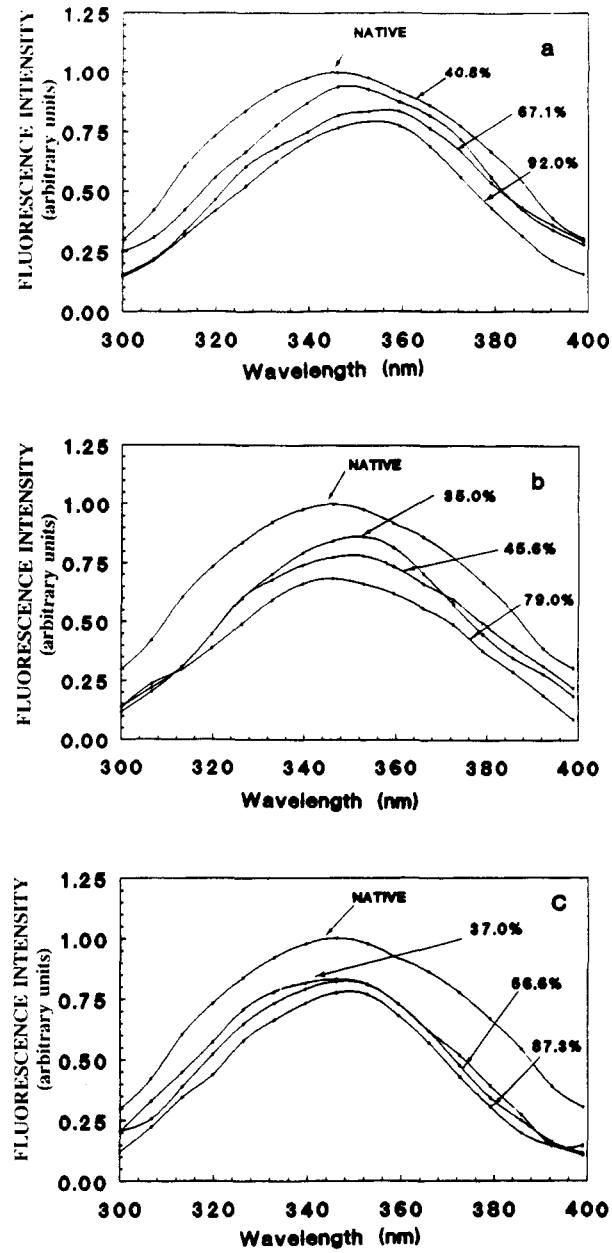

Figure 3. Fluorescence emission spectra of native and modified casein: succinylated (a), alkylated (b), and acetylated (c). Samples of 0.02% concentration in 0.1 M phosphate buffer, pH 7.01, were excited at 295 nm.

and Salahuddin (1978), using solvent perturbation, obtained a significant increase in the number of exposed tryptophans and tyrosines in highly acetylated ovalbumin.

In this study, spectra of acetylated casein, WPI, and BSA showed progressive peak shifts, although to a lower extent than those of the succinylated proteins, with increased degrees of modification. Alkylated WPI showed a shorter red shift than their succinylated counterparts at all degrees of modifications, while at 71.4% alkylation BSA did not show any difference from the native. Alkylated casein, on the other hand, showed a distinguished trend, i.e., enhanced shifts at lower degrees of modification followed by a blue shift thereafter. It seems that the unfolding and the disorganization of the interior hydrophobic regions of casein were followed by some refolding mechanism and chain associations leading to lower exposure of the tryptophan groups to the polar medium of the solvent and thus to a blue shift in the fluorescence spectra.

Circular Dichroic Spectral Properties. Circular dichroic (CD) spectral properties of native and acylated proteins were determined in the far-UV region (Figures 5–7). This region of the light spectrum is known for its inherent reliability in reflecting changes in the secondary structure of proteins due to folding and unfolding. It has also the advantage of being insensitive to nonchromophoric side chain variations. For each band, changes in the integrated intensity and peak shift were recorded and used as experimental parameters of the electronic transition of the protein macromolecules. The CD spectrum of native BSA shows the dominance of two negative peaks, at 208 and around 222 nm, a strong positive peak in the vicinity of 195 nm, and a broad shoulder that extends from 208 to about 240 nm. These features are sufficient indicators of a highly ordered structure, most probably of the $\alpha+\beta$ or α/β types, with considerable α-helical content. However, the depth of the 208-nm band and the relative shallowness of the 222-nm band are normally characteristics of an $\alpha+\beta$ structure. These results are in good agreement with the

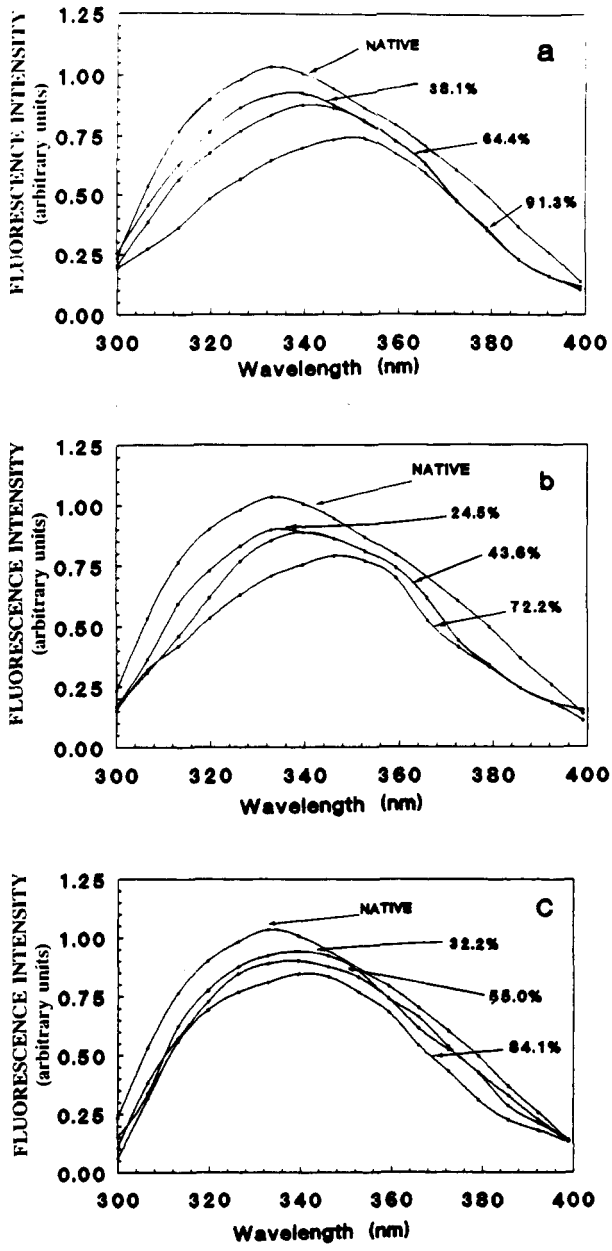

Figure 4. Fluorescence emission spectra of native and modified WPI: succinylated (a), alkylated (b), and acetylated (c). Samples of 0.02% concentration in 0.1 M phosphate buffer, pH 7.01, were excited at 295 nm.

CD spectral properties described by Peters (1985), who suggested that BSA has 50% of its chains in the α-helical conformation. Upon acetylation and succinylation, BSA spectra showed only a decrease in band intensity (negative ellipticity) at all wavelengths of the far-UV spectrum, without any significant shifts in the location of the CD peaks. The drop in the 195-nm band for all of the modification levels attained is an indicator of some loss in the α-character. The broadness of the shoulder implies increased β-structure content. No evidence for any significant trend toward randomness can be predicted from these profiles. Alkylated BSA (Figure 5b), on the other hand, showed insignificant change whether in the location or in the intensity of the bands, which leads to the conclusion that this treatment had the least effect on changes in the secondary properties of this protein.

Comparison of the CD profiles with the fluorescence patterns of acylated BSA showed a low correlation between these two parameters. Changes in fluorescence emission spectral peaks are usually an indication of the modification

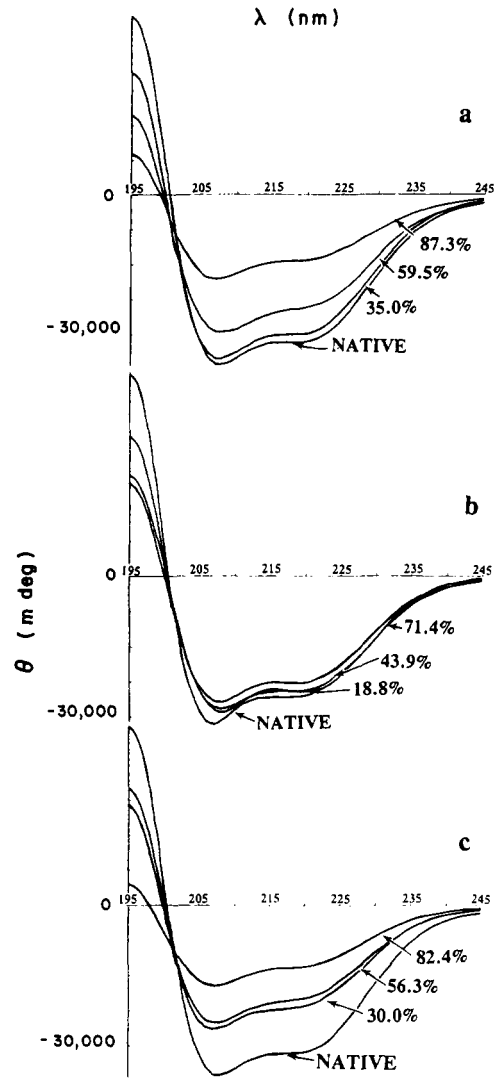

Figure 5. Far-UV circular dichroic spectra of native and modified BSA: succinylated (a), alkylated (b), and acetylated (c). Spectra were recorded for proteins 0.02% in phosphate buffer, pH 7.01.

of the tertiary structure of the protein as due to some perturbation of the tryptophan environment, while those of the CD reflect modifications in the secondary structure. The decrease in band intensity may be an indication of some changes in the tertiary structure of proteins. For the specific case of BSA, however, the involvement of disulfide bridges in the inherent optical properties of the protein and the physical constraints imposed on the protein's internal conformation are of considerable importance and may be responsible for the persistence of the native-like secondary structure. These results show, in general, that only the tertiary structure of BSA was affected by the modifications employed, while the secondary structure did not show any significant change.

CD spectra of native casein showed a highly flexible profile as evidenced in the sharp negative peak in the vicinity of 185 nm and a featureless broad shoulder afterward (Figure 6). The slim shoulder that extends from 205 to 230 nm may be due to some residual β-structure. Casein, a proline-rich protein, is an ideal representation of randomly coiled polymers with no helical structure (Slattery, 1976). These features may explain the high degree of modification attained by casein as compared to those of BSA and WPI. A progressive tendency toward decreased negative ellipticities was obtained for all of the acylated treatments accompanied by slight wavelength

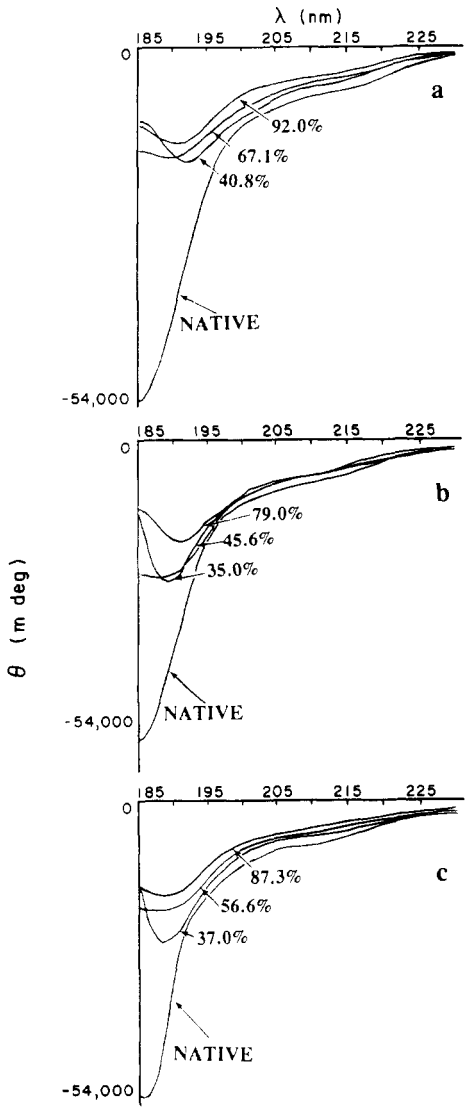

Figure 6. Far-UV circular dichroic spectra of native and modified casein: succinylated (a), alkylated (b), and acetylated (c). Spectra were recorded for proteins 0.02% in phosphate buffer, pH 7.01.

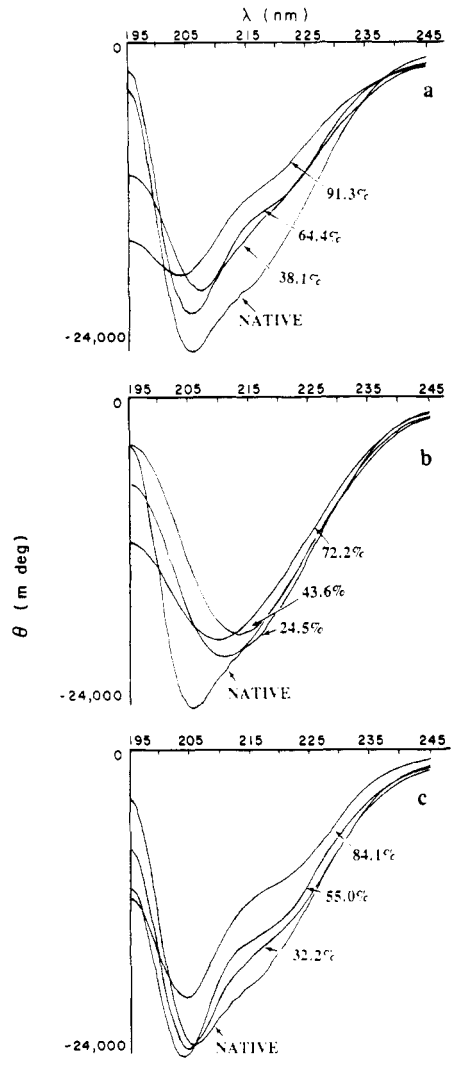

Figure 7. Far-UV circular dichroic spectra of native and modified WPI: succinylated (a), alkylated (b), and acetylated (c). Spectra were recorded for proteins 0.02% in phosphate buffer, pH 7.01.

shifts toward ordered conformations. Acylated caseins were found to experience a significant change in both intensity of the peak and its location, reflecting increased degree of order, possibly due to enhanced hydrophobic interactions between adjacent chains. This effect was more pronounced in alkylated caseins which showed a considerable tendency toward ordered conformations, which may be highly correlated to changes in hydrophobicity and fluorescence blue shifts.

CD spectra of native and modified WPI are presented in Figures 7. Native WPI showed a very strong negative peak at 208 nm and a weaker one at 220 nm, indicating a strong dominance of the α-character. Apparently, the crossover from positive to negative regions of the ellipticity occurred at less than 195 nm, which is another implication of the dominance of the α-character. In fact, the large overlap of the 208- and 222-nm bands and the corresponding drift in both peaks toward a single one are well-known features of $\alpha+\beta$ structures. The skewness of this mixed band toward the 208-nm side is indicative of the prevalence of pleated antiparallel β-sheets. Upon acylation, the secondary structure of WPI showed a wide variation in the circular dichroic responses. Succinylation and acetylation (parts a and c of Figure 7, respectively) resulted in a pronounced tendency toward randomness at the expense of helicity at all levels of modification. This effect was more obvious with succinylated WPI. A supporting evidence for the loss in the content of helical/ordered species is the significant drop in the 195-nm peak. These observations are in good agreement with the fluorescence emission spectra and the hydrophobicity of modified WPI. An interesting feature of acylated WPI is the case of alkylation (Figure 7b), where increased degree of alkylation resulted in a progressive increase in the content of ordered structures as reflected in the significant shifts toward longer wavelengths. At 72.2% alkylation, the 208-nm peak was found to shift to 216 nm, implying the dominance of a folded and highly ordered substructure. It appears that both the secondary and the tertiary structures of whey proteins were affected by acylation, although not to the same extent.

Nakagawa et al. (1972), using CD analysis, reported a significant loss in the content of β-structure of human serum IgG upon acylation with citraconic anhydride. However, acylation of human serum albumin (HSA) by various reagents was found not to affect its α-helical content. Batra et al. (1990) found that the secondary structure of ovalbumin remained resistant to acylation until about 15 of the 20 lysine residues were modified. Further modification caused a change in the secondary structure of the protein. They predicted that the instability of the secondary structure involved electrostatic as

well as steric hindrance caused by the added bulky acyl groups. Habeeb (1967) reported that succinylation of the first 30–40 lysine residues in BSA caused a small change in its Stokes radius, while succinylation of the remaining amino groups caused a dramatic increase in this parameter. Shimizu et al. (1985) found that although surface hydrophobicity of β-lactoglobulin significantly changed when the pH was lowered, no significant difference in the secondary structure of this protein was observed.

In summary, it is evident from this work that the effect of acylation on the physicochemical properties of a given protein cannot be explained only on the basis of the nature of the acylating group but should rather take into consideration the intrinsic properties of the modified proteins and the assembly of its subunits.

The structurally flexible protein, casein, was found to attain the highest degree of modification for all of the treatments employed; however, changes in its spectral properties did not follow the same trend. In fact, changes in the conformational structure of casein, upon acylation, in terms of the onset of denaturation were less significant. Increased hydrophobic interactions of casein subunits at higher degrees of alkylation were found to result in depressed surface hydrophobicity (as measured by ANS probe), blue-shifted emission fluorescence spectra, and an obvious tendency toward increased order.

The globular proteins, BSA and WPI, were found to experience significant degrees of denaturation upon acylation, which may lead to the conclusion that proteins with high content of tertiary structure can benefit from these modifications by means of enhanced flexibility, as reflected in their enhanced fluorescence red shifts and improved hydrophobicities. These indicators of molecular flexibility are expected to be paralleled by a significant tendency toward disorder. This mechanism was well-pronounced in WPI but not in BSA. Despite the fact that BSA is characterized by a flexible conformation, its CD profile suggests the interference of the disulfide bridges in their CD spectral properties, as is the case with most highly S–S cross-linked proteins.

Regarding the effect of the acyl group per se on the conformational structure of the proteins, it appears that despite the favorable effects of increased hydrophobicity on the conformational flexibility of a given protein, a balancing effect should be established between hydrophobic interactions and chain–chain attractive forces. When the latter forces dominate, the protein is expected to regain its folded native structure; even for randomly coiled proteins, this effect might result in enhanced order of the molecule at the expense of flexibility and disorder.

This study may, in addition, have presented an evidence that electrostatic interactions, upon acylation, cannot be used as sole evidence for changes in the physicochemical properties of proteins. Acetylation treatment, which is expected to neutralize the charge balance of the proteins, was found to affect the conformational structure of the three proteins and to enhance their denaturation.

Finally, in the evaluation of the conformational structural characteristics of a given protein, information on both its secondary and tertiary structures is required. Also, the assembly of its subunits and their interactions should be taken into consideration.

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LITERATURE CITED

Akita, A. M.; Nakai, S. Lipophylization of β-lactoglobulin. Effect on hydrophobicity, conformation and surface functional properties. *J. Food Sci.* **1980**, *55* (3), 711.

Ball, H. R.; Winn, M. Acylation of egg white proteins with acetic anhydride and succinic anhydride. *Poult. Sci.* **1982**, *61*, 620.

Batra, P.; Roebuck, M.; Uterht, D. Effect of lysine modification on the secondary structure of ovalbumin. *J. Protein Chem.* **1990**, *9* (1), 37.

Bjarnson, J.; Carpenter, K. J. Mechanism of heat damage in proteins. I. Models with acylated lysine residues. *Br. J. Nutr.* **1969**, *23*, 859.

Choi, Y. R.; Lusan, E. W.; Rhee, K. C. Succinylation of cottonseed flour: Effect on the functional properties of protein isolates prepared from modified flour. *J. Food Sci.* **1981**, *46*, 954.

Damodaran, M.; Kinsella, J. E. Interaction of carbonyls with soy protein: conformational effects. *J. Agric. Food Chem.* **1981**, *30*, 812.

Fields, R. Rapid determination of amino groups with TNBS. In *Methods Enzymol.* **1970**, *25B*, 464.

Franzen, K. L.; Kinsella, J. E. Functional properties of succinylated and acetylated soy proteins. *J. Agric. Food Chem.* **1976**, *24*, 788.

Geugen, J.; Bollecker, S.; Schwenke, K.; Raab, B. Effect of succinylation on some physicochemical and functional properties of the 12S storage proteins from rapeseed (*Brassica rapus L.*) *J. Agric. Food Chem.* **1990**, *38*, 61.

Groninger, H., Jr. Preparation and properties of succinylated fish myofibrillar protein. *J. Agric. Food Chem.* **1973**, *21*, 978.

Groninger, H. S.; Miller, R. Preparation and aeration properties of an enzyme-modified succinylated fish protein. *J. Food Sci.* **1975**, *40*, 327.

Habeeb, A. F. Quantitation of conformational changes on chemical modification of proteins: Use of succinylated proteins as a model. *Arch. Biochem. Biophys.* **1967**, *121*, 652.

Habeeb, A. F.; Cassidy, H. G.; Singer, M. Molecular structural effects produced in proteins by reaction with succinic anhydride. *Biochim. Biophys. Acta* **1958**, *29*, 587.

Haque, Z.; Kito, M. Lipophilization of α_{s1}-casein. 2. Conformational and functional effect. *J. Agric. Food Chem.* **1983**, *31*, 1231.

Hayakawa, S.; Nakai, S. Relationship of hydrophobicity and net charge to the solubility of milk and soy proteins. *J. Food Sci.* **1985**, *50*, 486.

Hoagland, P. Acetylated-caseins: Electrostatic interactions and aggregation. *J. Dairy Sci.* **1966**, *49*, 783.

Kim, S. H. Kinsella, J. E. Effects of progressive succinylation on some molecular properties of soy glycinin. *Cereal Chem.* **1986**, *63* (4), 342.

Kitabatake, N.; Cuq, J. L.; Cheftel, J. C. Covalent binding of glycosyl residues to β-lactoglobulin: Effect on solubility and heat stability. *J. Agric. Food Chem.* **1985**, *33*, 125.

Lakkis, J.; Villota, R. Comparative performance of chemically- and enzymatically-modified whey proteins. *Food Chem.* **1992**, *43*, 93.

Lee, H. S.; Sen, L. C.; Clifford, A. J.; Whitaker, J. R.; Feeney, R. E. Effect of reductive alkylation of the α-amino groups of lysyl residues of casein on its nutritive value in rats. *J. Nutr.* **1978**, *108*, 687.

Li-Chan, E.; Helbig, N.; Holbek, E.; Chau, S.; Nakai, S. J. Covalent attachment of lysine to wheat gluten for nutritional improvement. *J. Agric. Food Chem.* **1979**, *27*, 877.

Ma, C. Y.; Holme, R. Effect of chemical modification on some physicochemical properties of heat coagulation of egg-albumin. *J. Food Sci.* **1982**, *47*, 454.

Means, M. A.; Feeney, R. E. Reductive alkylation of amino groups in proteins. *Biochemistry* **1968**, *7*, 2192.

Means, M. A.; Feeney, R. E. In *Chemical Modification of Proteins*; Holden-Day: San Francisco, 1971.

Melnychym, P.; Stapely, R. Acylated proteins for coffee whitener formulations, U.S. Pat. 3 764 711, 1973 (to Carnation Co.).

Montejano, J. G.; Hamann, D. D.; Ball, H. R.; Lanier, T. C. Thermally induced gelation of native and modified egg white-rheological changes during processing: Final strength and microstructures. *J. Food Sci.* **1984**, *49*, 1249.

Nakagawa; Capetillo, S.; Jirgensons, B. Effect of chemical modification of lysine residues on the conformation of human Ig G. *J. Biol. Chem.* **1972**, *247*, 5703.

Peters, T. Jr. Serum albumins. *Adv. Protein Chem.* **1985**, *37*, 161.

Puigserver, A. J.; Sen, L. C.; Clifford, A. J.; Feeney, R. E.; Whitaker, J. R. Covalent attachment of amino acids to casein. I. Chemical modification and rates of in vitro enzymatic hydrolysis. *J. Agric. Food Chem.* **1979**, *27*, 1099.

Qasim, M. A.; Salahuddin, A. Changes in conformation and immunological activity of ovalbumin during its modification with different acid anhydrides. *Biophys. Biochim. Acta* **1978**, *536*, 50.

Rao, A. J.; Rao, M. S. Effect of succinylation on the oligomeric structure of glycinin. *Int. J. Pept. Protein Res.* **1979**, *14*, 307.

Rauschal, E. J.; Linow, K. J.; Pahtz, W.; Schwenke, K. D. *Nahrung* **1981**, *25*, 241.

Schwenke, K. D.; Rauschal, E. J. Functional properties of plant proteins. Part 6. Some functional properties of succinylated protein isolates from sunflower seed (*Helianthus annus L.*). *Nahrung* **1980**, *27*, 1015.

Schwenke, K. D.; Linow, K. J.; Zirwer, D. Modification of the oligomeric structure of 11S globulin from sunflower (*Helianthus annus L.*) and rape (*Brassica napus L.*) seeds by succinylation. *Nahrung* **1986**, *30*, 263.

Sen, L.; Lee, H.; Feeney, R.; Whitaker, J. In vitro digestibility and functional properties of chemically modified casein. *J. Agric. Food Chem.* **1981**, *29*, 348.

Shetty, J. K.; Kinsella, J. E. Reversible modification of lysine: Separation of proteins and nucleic acids in yeast. *Adv. Chem. Ser.* **1982**, *No. 198*, 169.

Shimizu, M.; Saito, M.; Yamauchi, K. Emulsifying and structural properties of β-lactoglobulin at different pHs. *Agric. Biol. Chem.* **1985**, *49*, 189.

Slattery, C. W. Casein micelle structure; an examination of models. *J. Dairy Sci.* **1976**, *59*, (9), 1547.

Voustinas, L. P.; Nakai, S. J. Covalent binding of methionine and tryptophan to soy protein. *J. Food Sci.* **1979**, *44*, 1205.

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