Grafting of Aliphatic and Aromatic Probes on Rapeseed 2S and 12S Proteins: Influence on Their Structural and Physicochemical Properties

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Lysyl residues of rapeseed napin (2S) and cruciferin (12S) were acylated and sulfamidated by means of anhydrides and sulfonyl chlorides, respectively. The secondary and tertiary structures as well as the surface hydrophobicity of the modified proteins were studied using circular dichroism, intrinsic fluorescence, and binding of anilinonaphthalenesulfonic acid. The results showed clearly that grafting of hydrophobic chains induced different structural modifications and surface hydrophobicities on the monomeric (2S) and on the hexameric (12S) proteins. Thus, the original structure of the 2S modified protein seemed to be preserved. Therefore, the surface hydrophobicity increased proportionally with the number of groups grafted. Conversely, after modification, 12S was shown to be expanded. As a result, hydrophobic regions were exposed, leading to a much greater hydrophobization of the protein surface. Acylation and sulfamidation appeared, therefore, to be good methods to hydrophobize efficiently the surface of the two proteins and thus might probably induce new functional properties.

Keywords: *Rapeseed proteins; acylation; sulfamidation; structure; hydrophobicity*

INTRODUCTION

The growth of world population induces an increasing demand not only for animal feeds and human foods but also for a better utilization of materials, space, and energy. Consequently, an emerging research area has been concerned with studies on the potential of agricultural crops for nonfood uses.

Seed proteins, which are commonly used for nutritional applications, also possess interesting potential as biopolymers for preparing glues or plastic-like materials (Rayas et al., 1997; Guéguen et al., 1998). These properties might generate an added-value utilization for some oilseed meal of lower nutritional quality. This is the case for rapeseed meal, the utilization of which is limited for nutritional purposes by the denaturation of proteins during oil processing and the presence of phytic acid and phenolic compounds. The rather high content of proteins in the rapeseed meal [40−45% dry matter (dm)] and the increasing production of rapeseed since 1987 in Europe and more generally in the world (7 million tonnes produced in 1970 and 28 million tonnes in 1991) are major reasons for developing alternative nonfood uses for these proteins. However, this development requires the improvement of the functional properties of the rapeseed proteins, which can be obtained through physical, enzymatic, or chemical treatments.

In food technology, the main tools for protein modification are physical (pressure, temperature) and enzymatic, as contrasted to chemical treatments, which are not often used because of reagent toxicity and formation of side-reaction products. Conversely, chemical tailoring of proteins has mainly been studied for nonfood uses not only to improve their functional properties but also to investigate structure−function relationships (Kinsella, 1976; Schwenke, 1978; Kinsella and Shetty, 1979; Wright and Bumstead, 1984; Wright, 1985; Caer et al., 1990; Chambers et al., 1990; Baniel et al., 1992; Colas et al., 1993; Subirade et al., 1994). Among the various chemical treatments, acylation, with acetic or succinic anhydride (Ma, 1984; Paulson and Tung, 1987), is widely used for improving the functional properties of plant proteins. Acetylation and succinylation have already been applied to wheat (Grant, 1973), soybean (Franzen and Kinsella, 1976), cottonseed and peanut (Beuchat, 1977), sunflower (Kabirullah and Wills, 1982), and pea proteins (Johnson and Brekke, 1983; Schwenke et al., 1990, 1993; Subirade et al., 1992). These kinds of modification have also been applied to the two main protein fractions of rapeseed, the neutral high molecular mass 12S globulin (cruciferin) and the basic low molecular mass 2S albumin (napin) fraction, especially by Schwenke and collaborators. The rapeseed 2S protein (napin) is a basic low molecular mass protein (∼14000 g/mol), with a high content of sulfur-containing amino acids, rich in helical conformation and built of a larger and a smaller disulfide bridged polypeptide. On the other hand, the 12S protein is a globulin protein built of six subunits, each of them composed of a larger and a smaller polypeptide chain. It is characterized by a sedimentation coefficient of 11−13S, a molecular mass of ∼300000 g/mol, and a high percentage of β-sheet conformation.

According to Schwenke (1990), increasing succinylation results in a step-by-step dissociation and unfolding

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of rapeseed 12S but, conversely, acylating 2S does not abolish its secondary or tertiary structure. Nitecka et al. (1986) showed that after succinylation, the intrinsic viscosity of 2S drops, its emulsifying properties and foaming capacity are not improved, and its foam stability decreases. After acetylation, Schwenke et al. (1991) noticed a stabilizing effect of the modification against heat-induced aggregation, a slight change in its foam capacity and stability, and a decrease in its emulsifying capacity. By succinylating cruciferin, Nitecka and Schwenke (1986) showed that up to 61% modification, foam capacity and stability as well as emulsifying activity and emulsion stability were either not or only slightly improved.

According to these previous results, only very limited improvement in the functional properties of rapeseed proteins occurred by acylation with acetic or succinic anhydride. However, no study has been made upon the effect of grafting more hydrophobic groups, such as longer aliphatic or aromatic chains, whereas the functional properties of proteins were shown to be related to their surface hydrophobicity (Kato and Nakai, 1980; Nakai, 1983; Shimizu et al., 1983) and structure (Damodaran and Song, 1988; Subirade et al., 1992). In consequence, the present work deals with the influence of such grafting on the structural characteristics (secondary and tertiary structures) and surface hydrophobicity of 2S and 12S rapeseed proteins. We originally developed the acylation with longer anhydrides and the sulfamidation with *p*-arylsulfonyl chlorides reacting on lysine residues in a former study on model proteins (Gerbanowski et al., 1999).

MATERIALS AND METHODS

Preparation of Rapeseed Proteins. The seeds (var. Lirajet), obtained from Bioraf Denmark within the framework of a FAIR European contract (CT 95-260), were dehulled and defatted in hexane. The protein extract was prepared by mixing (2 h, room temperature) the resulting flour with sodium phosphate buffer (22 mM, pH 7) containing 3‰ sodium bisulfite used as reductive agent to limit the oxidation of phenolic compounds and thus their binding to the proteins (Petit et al., 1979). The mixture was centrifuged at 30000*g* for 20 min, and the supernatant was loaded on a chromatographic gel filtration column (Sephadex GH 25, Amersham Pharmacia) equilibrated with the extraction buffer. The proteins were collected and then separated by cation exchange chromatography (SP-Sepharose Big Bead, Amersham Pharmacia) using the extraction buffer. Rapeseed 12S proteins were not adsorbed on the column, and the 2S proteins were eluted with an NaCl gradient. Salts were eliminated by gel filtration (column GH 25, Amicon Millipore) against 0.1% ammonium carbonate buffer. The samples were then lyophilized. A verification of the total elimination of ammonium carbonate after lyophilization was made. The protein content of samples was then estimated according to Kjeldhal's method using a conversion factor N/protein = 5.5 for rapeseed proteins (Mosse, 1990). The samples of 2S and 12S contained 90 and 84% of proteins, respectively, the remaining part being constituted of the nondialyzed salts.

Protein Modification. Acylation and sulfamidation were carried out on 2S and 12S proteins as previously described (Gerbanowski et al., 1999). Three equivalents of anhydride and 2 equiv of arylsulfonyl chloride per lysine residue were added dropwise to the protein solution (2 mg/mL in water) to reach the maximum level of modification. Lower degrees of modification were obtained by decreasing the ratio of the electrophilic reagent per lysine residue. The pH was kept at 10.5 during the reaction by a controlled and continuous addition of 0.5 N NaOH using an automatic pH-stat titration device. After the end of the reaction (when the pH of the medium remained constant), salts and excess reagents were removed by dialysis against distilled water. The protein solution was then lyophilized. Three washings of the freeze-dried protein sample (10 mg) in hexane (5 mL) were performed to remove the side-reaction products (fatty acids, aryl sulfonates) noncovalently bound to the protein.

Determination of the Degree of Modification. The proportion of free amino groups was determined as previously described (Gerbanowski et al., 1999) according to the modified *o*-phthaldialdehyde (OPA) method (Frister et al., 1988).

The percentage of lysine modification was expressed as $(AG_{nm} - AG_m)/AG_{nm} \times 100$, AG_{nm} and AG_m being the numbers of free amino groups in nonmodified and modified protein, respectively. The experimental values found for AG_{nm} were in agreement with the number of 2S and 12S amino groups calculated from the sequence.

Circular Dichroism (CD) Measurements. The secondary and tertiary structures of native and modified proteins were studied using CD spectra in far-UV (from 190 to 250 nm) and near-UV (from 250 to 320 nm).

The measurements were performed on a dichrograph CD185 (Roussel-Jouan, Paris) at 25 °C in standard buffer (0.1 M sodium phosphate, pH 7) as described previously (Gerbanowski et al., 1999) using protein concentrations of 2.5 and 5 mg/mL in the far-UV (0.1 mm cell) and near-UV (2 mm cell) regions, respectively. The estimation of the secondary structure was carried out by using the method of Provencher and Glöckner (1981). The reported CD spectra in far- or near-UV were the result of 10 scans.

A solution of *d*-camphor-10-sulfonic acid (0.6 mg/mL), which has a positive CD (a molar ellipticity of 7260 deg cm^2 $dmol^{-1}$) at 290.5 nm (Cassim and Yang, 1969), was used to calibrate the spectropolarimeter.

Intrinsic Fluorescence. The intrinsic fluorescence emission spectra between 305 and 445 nm were recorded with a Fluoromax Spex (Jobin Yvon) at 25 °C using an excitation wavelength at 295 nm and quartz optical cells of 10 mm path length. Excitation and emission bandwidths were set at 5 nm. The protein concentrations were adjusted exactly to 0.05 mg/mL (0.1 M sodium phosphate buffer, pH 7) to obtain a 280 nm absorbance value below 0.1 compatible with fluorescence measurements (avoidance of inner filter effects). Corrections for the fluorescence of the buffer blanks were made.

Surface Hydrophobicity. The surface hydrophobicity of 2S and 12S proteins, native and modified, was studied by fluorescence spectroscopy following the binding of a hydrophobic probe, 8-anilino-1-naphthalenesulfonic acid (ANS), by the protein (Gerbanowski et al., 1999).

The binding parameter values between ANS and the protein, the number of binding sites n, and the association constant K_a, determined by Scatchard's method (Scatchard et al., 1957) or Klotz's method (Klotz and Hunston, 1971), enabled the surface hydrophobicity of each protein to be evaluated. This study was performed using the general procedure of Cardamone and Puri (1992). First, a calibration factor (ΔF_{max}) for each protein, corresponding to the enhancement of fluorescence due to the binding of one molecule of ANS, was determined by titration of a fixed concentration of ANS (1 mL of 1 μM in phosphate buffer) by successive addition of protein solution (1.5 μM). By plotting the inverse of the fluorescence intensity versus the inverse of the protein concentration, an estimation of the fluorescence increase per micromolarity unit of ANS bound (ΔF_{max}) can be obtained using the ordinate intercept as the extrapolation of the resulting straight line to infinite protein concentration.

Knowing this factor, the enhancement of fluorescence observed in a second set of experiments, consisting of the protein titration (0.5 μM protein in phosphate buffer) by ANS (253 μM in phosphate buffer), led to the number of ANS molecules bound to the protein. Using Scatchard's or Klotz's method, the number of ANS binding sites and the affinity constant could be evaluated.

Table 1. Percentage of Modification of Rapeseed 2S and 12S Proteins Using 3 equiv of Anhydride and 2 equiv of Sulfonyl Chloride per Lysine Residue[a]

reagent	% of modification (SD) 2S	12S
acetic anhydride (R = C2)	95 (0.45)	62 (0.52)
propionic anhydride (R = C3)	65 (0.60)	49 (0.52)
butyric anhydride (R = C4)	93 (0.83)	64 (0.63)
hexanoic anhydride (R = C6)	89 (0.21)	52 (0.32)
p-toluenesulfonyl chloride (R = C1)	43 (0.83)	46 (1.8)
ethylbenzenesulfonyl chloride (R = C2)	37 (0.50)	43 (0.95)

[a] These values were determined according to the modified *o*-phthaldialdehyde (OPA) method (Fister et al., 1988) and expressed as percentage of lysine residues modified. Experimental reaction conditions: water, room temperature, pH 10.5. (SD), standard deviation of two replicate experiments.

RESULTS

Extent of Chemical Modification. The complete conversion of a model molecule (*N*-α-acetyllysine) was achieved by using 3 equiv of anhydride and 2 equiv of arylsulfonyl chloride per *N*-α-acetyllysine (Gerbanowski et al., 1999). These conditions of modification were applied successfully to rapeseed proteins.

Treatment of napin and cruciferin with a small excess of anhydride (3 equiv per lysine residue) led to a rather high level of acylation of the ϵ-amino group of lysine as shown in Table 1. The levels of acylation were higher for 2S than for 12S, and short anhydrides were more reactive than longer ones. Thus, 95% of lysine residues in 2S were acylated using acetic anhydride, whereas 89% were modified using hexanoic anhydride. These two reagents acylated, respectively, 62 and 52% of lysine residues in 12S.

The percentages of sulfamidation, using 2 equiv of reagent per lysine, were lower than those of acylation. Values of ∼40% were obtained whatever the reagent used (*p*-toluenesulfonyl chloride or ethylbenzenesulfonyl chloride) both with 2S and with 12S.

Influence of Modification on the Secondary Structure of Rapeseed Proteins. A Far-UV CD Study. The far-UV spectrum of native 2S had two negative minima at 208 and 222 nm and a maximum at 190−195 nm, which is typical of the α+β class protein (Figure 1) with 41% of α-helix and 16% of β-sheet (Table 2).

Upon exhaustive acylation, 2S spectra showed only a decrease in band intensity at all wavelengths of the far-UV CD without any significant shift of the peaks. This decrease was much greater for 2S modified with hexanoic anhydride than for other anhydrides. It revealed a small decrease in the α-helix content and a large increase in the β-sheet content (Table 2).

Sulfamidation also created a decrease in the molecular ellipticity, much greater for the *p*-toluenesulfonyl modification than for the ethylbenzenesulfonyl chloride one (Figure 1). The α-helix structure decreased drastically in this protein, and its secondary structure was composed mainly of β-sheet and random coil. For 2S sulfamidated with ethylbenzenesulfonyl chloride, the α-helix and random coil structures decreased, whereas the β-sheet one increased remarkably (Table 2).

The spectrum obtained with native 12S showed broad negative peaks in the 210−215 nm region (Figure 2), characteristic of predominantly β-sheet proteins. This was further substantiated by the fact that the spectrum also showed a positive peak at 194 nm associated with

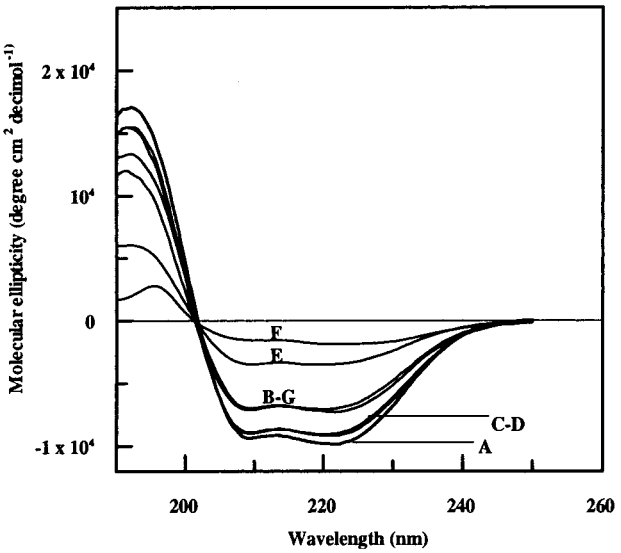

Figure 1. Far-UV CD spectra of native and modified 2S: (A) native 2S; (B) 2S/acetic anhydride (95%); (C) 2S/propionic anhydride (65%); (D) 2S/butyric anhydride (93%); (E) 2S/hexanoic anhydride (89%); (F) 2S/*p*-toluenesulfonyl chloride (43%); (G) 2S/ethylbenzenesulfonyl chloride (37%). The spectra were performed in a quartz cell (0.1 mm) at 25 °C in standard buffer (0.1 M sodium phosphate, pH 7) using protein concentrations of 2.5 mg/mL. Appropriate baseline subtraction was done.

the β-sheet structure. A very weak shoulder at 221 nm also present in the spectrum indicated the presence of very little α-helix in its secondary structure. This was confirmed by using the method by Provencher et al. (1981), which revealed a content of 10% α-helix and 69% β-sheet (Table 3).

After acylation, a decrease in the molecular ellipticity in the 190−195 and 210−215 nm regions was noticed. The negative minimum at 210 nm was also displaced toward a shorter wavelength, indicating an important change in the secondary structure of the protein after acylation. In fact, the calculation of secondary structure showed a slight but significant decrease in the α-helix content (10% for the native and 5−7% for the acylated proteins) and an increase in the remainder structure for the acylated proteins. On the other hand, sulfamidation induced a complete loss of the α-helix structure and an increase in the remainder structure (Table 3) confirmed by a large decrease in peak intensities (Figure 2).

Influence of Modification on the Tertiary Structure of Rapeseed Proteins. (A) A Near-UV CD Study. The near-UV spectra of acylated 2S are quite similar to the native spectrum except for 2S acylated with hexanoic anhydride (Figure 3). The spectra of sulfamidated proteins are also very different, with a shift of peaks for 2S sulfamidated with ethylbenzenesulfonyl chloride and a disappearance of some peaks for 2S sulfamidated with *p*-toluenesulfonyl chloride (Figure 3). These spectra highlight some conformational changes, affecting the tertiary structure of the protein, induced by the grafting of long aliphatic or aromatic chains.

The near-UV CD spectra of acylated and sulfamidated 12S are shown in Figure 4. 12S acylated and sulfamidated with *p*-toluenesulfonyl chloride showed a decrease in molecular ellipticity compared to native 12S, indicating slight conformational changes. Conversely, the spectrum of 12S sulfamidated with ethylbenzenesulfonyl chloride showed an increase in molecular ellipticity

Table 2. Composition of the Secondary Structure of Native and Modified 2S Obtained from Far-UV CD Spectra (Protein Concentration = 2.5 mg/mL in 0.1 M Phosphate Buffer, pH 7, 0.1 mm Cell, 25 °C) Using the Method of Provencher and Glöckner (1981)

protein	α-helix (%)	β-sheet (%)	remainder (%)
native 2S	41 (±0.005)	16 (±1)	43 (±1)
2S/acetic anhydride (95%)	30 (±1)	33 (±1)	37 (±2)
2S/propionic anhydride (65%)	38 (±0.004)	21 (±1)	41 (±1)
2S/butyric anhydride (93%)	39 (±0.005)	22 (±1)	40 (±1)
2S/hexanoic anhydride (89%)	12 (±1)	50 (±1)	39 (±2)
2S/*p*-toluenesulfonyl chloride (43%)	9 (±1)	55 (±1)	37 (±2)
2S/ethylbenzenesulfonyl chloride (37%)	23 (±1)	40 (±1)	36 (±2)

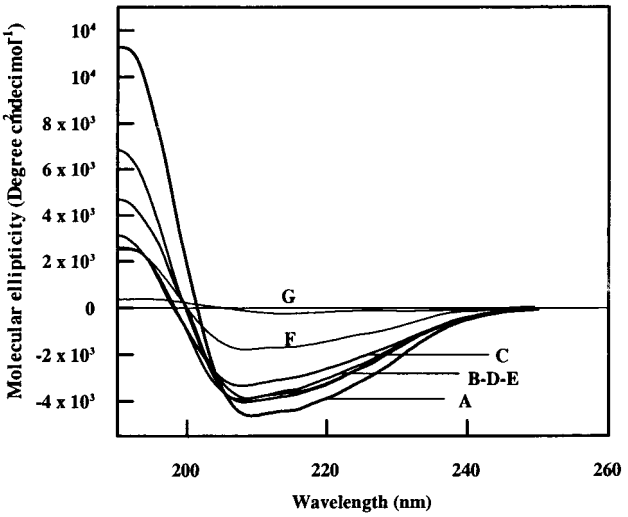

Figure 2. Far-UV CD spectra of native and modified 12S: (A) native 12S; (B) 12S/acetic anhydride (62%); (C) 12S/propionic anhydride (49%); (D) 12S/butyric anhydride (64%); (E) 12S/hexanoic anhydride (52%); (F) 2S/*p*-toluenesulfonyl chloride (47%); (G) 2S/ethylbenzenesulfonyl chloride (43%). The spectra were recorded in a quartz cell (0.1 mm) at 25 °C in standard buffer (0.1 M sodium phosphate, pH 7) using protein concentrations of 2.5 mg/mL. Appropriate baseline subtraction was done.

and a displacement of CD bands toward shorter wavelength. Thus, the grafting of these aromatic chains completely perturbed the initial conformation of the protein.

(B) An Intrinsic Fluorescence Study. The maximum emission wavelength for the native 2S was 333 nm. After modification with butyric or hexanoic anhydride, there was a progressive blue shift of the maximum emission from 333 nm toward 325 nm as a function of the level of modification and of the length of the chain grafted (Figure 5). These results revealed some tryptophan (Trp) environment alteration probably due to conformational changes, that is, alteration of the tertiary structure.

The maximum emission located at 328 nm for native protein 12S decreased in intensity even after a low level of acylation with butyric anhydride (21%) (Figure 6). At a higher level of modification (70%), the effect was much greater and a red shift of the maximum emission was observed. Acylation with longer chains had the same effect but even more marked. The decrease of fluorescence intensity and red shift may be due to exposure of tryptophan residues or a slight turbidity development. This phenomenon, starting at a low level of modification, increased at higher grafting rates.

As previously explained (Gerbanowski et al., 1999), sulfamidated protein spectra cannot be used to detect conformational changes because the emission observed after an excitation at 295 nm is not induced by the tryptophan but by the aromatic rings grafted (data not shown).

Influence of Modification on Surface Hydrophobicity of Rapeseed Proteins. Native 2S bound two molecules of ANS with an association constant of 1.7^{E+03} M^{-1}. The grafting of butyric chains resulted in the fixation of four and five ANS molecules for 23 and 94% modification, respectively (Figure 7). The maximum emission wavelength of the protein−ANS complex was blue shifted from 505 to 503 and 470 nm for low and high levels of acylation, respectively, indicating a progressive hydrophobization of the bound ANS environment (Slavik, 1982) as a function of the increasing number of grafted chains. Acylation with a longer aliphatic chain (hexanoic) increased 4-fold the number of ANS molecules bound. In this case, the latter also increased with the number of lysine residues modified. A blue shift in the maximum emission was also noticed. As previously observed for bovine serum albumin (BSA) (Gerbanowski et al., 1999), sulfamidation did not induce many new binding sites. Only one new binding site was detected on 2S sulfamidated with *p*-toluenesulfonyl chloride, but the blue shift observed was very large, indicating a hydrophobization of the ANS environment.

Native 12S bound eight molecules of ANS with an association constant of 2^{E+04} M^{-1} and a maximum emission of 466 nm. After acylation, the number of bound ANS molecules increased considerably with the level of modification (Figure 8) but, conversely, the length of the aliphatic chain grafted did not seem to exert an influence. A molecule of 12S modified at 61% with butyric anhydride bound 65 ANS molecules, and a molecule acylated at 50% with hexanoic anhydride bound 62 ANS molecules. In each case, a significant blue shift was noticed. Sulfamidation also created numerous new binding sites.

DISCUSSION

Both acylation and sulfamidation of amino groups were successfully performed for rapeseed 2S and 12S proteins.

As observed for the acylation of rapeseed proteins with succinic anhydride (Nitecka and Schwenke, 1986), in the same reaction conditions, the levels of modification reached were lower for 12S than for 2S. Only in the presence of an extremely high excess of reagent (100-fold excess) could 90% of the amino groups of rapeseed 12S be succinylated (Schwenke et al., 1986). A similar limitation has already been observed for some other plant globulins such as the 11S protein from peanut (Shetty and Rao, 1978) or sunflower seed (Schwenke et al., 1985), for which the highest level of blocking was 85%.

The reason for the incomplete reaction might be understood by considering the spatial structure of

Table 3. Composition of the Secondary Structure of Native and Modified 12S Obtained from Far-UV CD Spectra (Protein Concentration = 2.5 mg/mL in 0.1 M Phosphate Buffer, pH 7, 0.1 mm Cell, 25 °C) Using the Method of Provencher and Glöckner (1981)

protein	α-helix (%)	β-sheet (%)	remainder (%)
native 12S	10 (±2)	69 (±2)	21 (±3)
12S/acetic anhydride (62%)	5 (±1)	58 (±1)	37 (±2)
12S/propionic anhydride (49%)	4 (±1)	60 (±1)	36 (±2)
12S/butyric anhydride (64%)	7 (±1)	65 (±1)	28 (±2)
12S/hexanoic anhydride (52%)	7 (±1)	58 (±1)	36 (±2)
12S/*p*-toluenesulfonyl chloride (46%)	0 (±1)	68 (±1)	32 (±1)
2S/ethylbenzenesulfonyl chloride (43%)	0	71 (±1)	29 (±1)

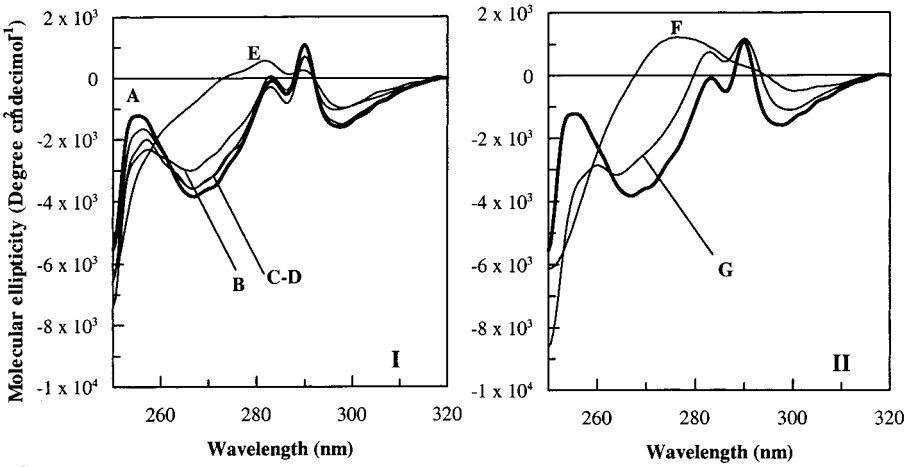

Figure 3. Near-UV CD spectra of native and modified 2S: (I) 2S acylated; (II) 2S sulfamidated; (A) native 2S; (B) 2S/acetic anhydride (95%); (C) 2S/propionic anhydride (65%); (D) 2S/butyric anhydride (93%); (E) 2S/hexanoic anhydride (89%); (F) 2S/*p*-toluenesulfonyl chloride (43%); (G) 2S/ethylbenzenesulfonyl chloride (37%). The spectra were recorded in a quartz cell (2 mm) at 25 °C in standard buffer (0.1 M sodium phosphate, pH 7) using protein concentrations of 5 mg/mL. Appropriate baseline subtraction was done.

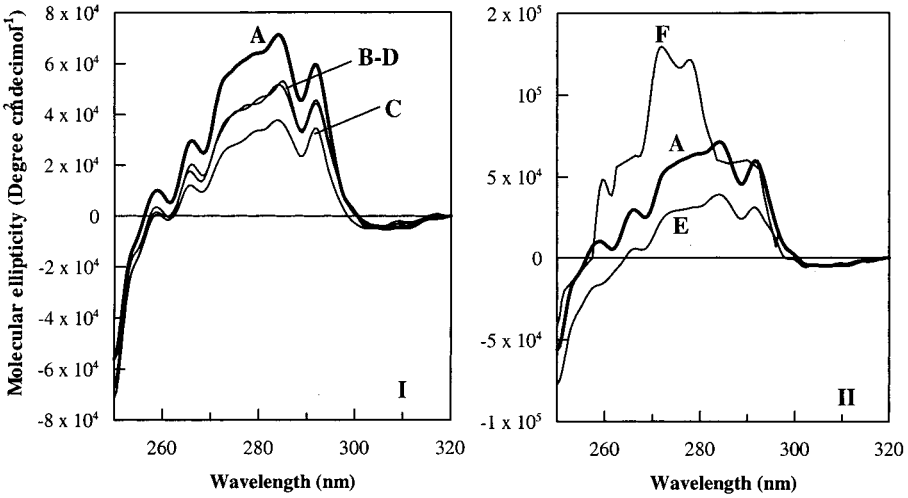

Figure 4. Near-UV CD spectra of native and modified 12S: (I) 12S acylated; (II) 12S sulfamidated; (A) native 12S; (B) 12S/acetic anhydride (62%); (C) 12S/propionic anhydride (49%); (D) 12S/butyric anhydride (64%); (E) 12S/hexanoic anhydride (52%); (F) 2S/*p*-toluenesulfonyl chloride (47%); (G) 2S/ethylbenzenesulfonyl chloride (43%). The spectra were recorded in a quartz cell (2 mm) at 25 °C in standard buffer (0.1 M sodium phosphate, pH 7) using protein concentrations of 5 mg/mL. Appropriate baseline subtraction was done.

globulins and the distribution of reactive amino groups, resulting in steric hindrance and a possible restraint of nucleophilic attack owing to proximity effects of adjacent residues. When an extremely large amount of reagent is used, the first amino groups modified probably induce an unfolding of the protein, making more amino groups accessible for further reactions.

Conversely, for 2S, high levels of acylation (90%) could be reached using only a small excess of anhydride, as shown by Schwenke (1990) with the napin protein using acetic or succinic anhydride. This result can be explained by the great accessibility of the lysine residues, mainly exposed at the protein surface.

In the case of acylation of both 2S and 12S, the percentage of lysine conversion decreased with the increasing chain length of the anhydride (Table 1). This effect could be due to steric hindrance induced by the reagent. As a result, the rate of the nucleophilic attack on lysine residues could be reduced using longer anhydrides.

Rapeseed protein functionalization with arylsulfonyl chloride led to lower levels of modification compared to

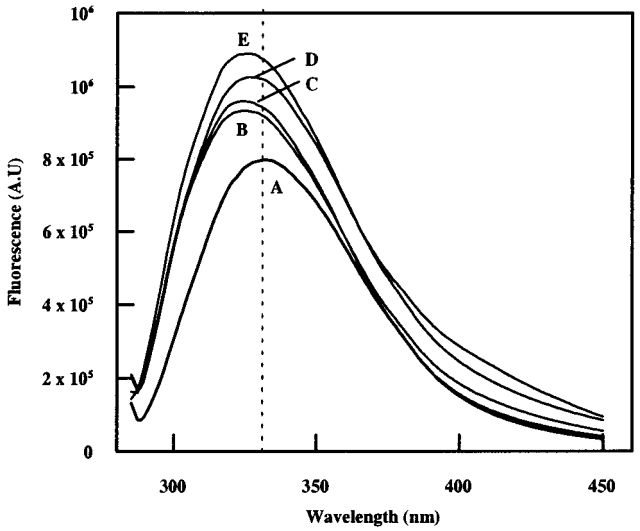

Figure 5. Intrinsic fluorescence spectra of native and acylated 2S: (A) native 2S; (B) 2S/butyric anhydride (23%); (C) 2S/butyric anhydride (93%); (D) 2S/hexanoic anhydride (24%); (E) 2S/hexanoic anhydride (89%). Spectra were recorded on protein solutions (0.05 mg/mL in 0.1 M sodium phosphate buffer, pH 7) at 25 °C using an excitation wavelength at 295 nm and quartz optical cells of 10 mm path length. Excitation and emission bandwidths were set at 5 nm. Corrections for the fluorescence of the blanks were made.

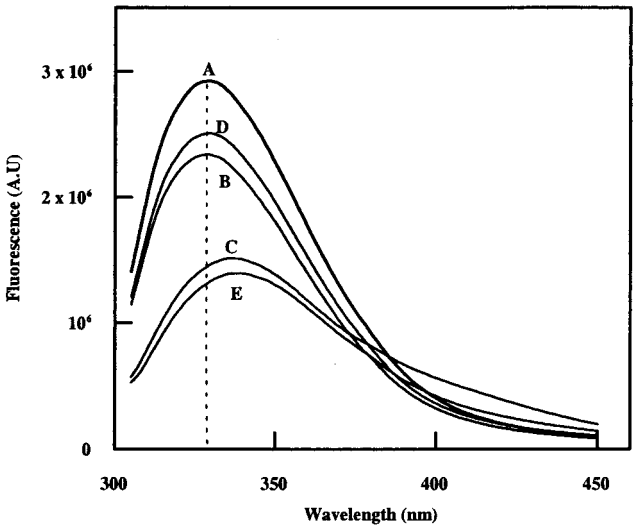

Figure 6. Intrinsic fluorescence spectra of native and acylated 12S: (A) native 12S; (B) 12S/butyric anhydride (21%); (C) 12S/butyric anhydride (70%); (D) 12S/hexanoic anhydride (23%); (E) 12S/hexanoic anhydride (52%). Spectra were recorded on protein solutions (0.05 mg/mL in 0.1 M sodium phosphate buffer, pH 7) at 25 °C using an excitation wavelength at 295 nm and quartz optical cells of 10 mm path length. Excitation and emission bandwidths were set at 5 nm. Corrections for the fluorescence of the blanks were made.

anhydrides. This result can also be explained by the relative bulkiness of the arylsulfonyl chlorides.

Studies of native and modified 2S and 12S using CD in far-UV, near-UV, and intrinsic fluorescence revealed some differences in the influence of grafting long and aromatic chains on the secondary and tertiary structures of the two proteins. These differences are probably due to the different structures and sizes of 2S and 12S.

The composition of the secondary structure of native 2S obtained in our study is in accordance with data previously published (Schwenke et al., 1988).

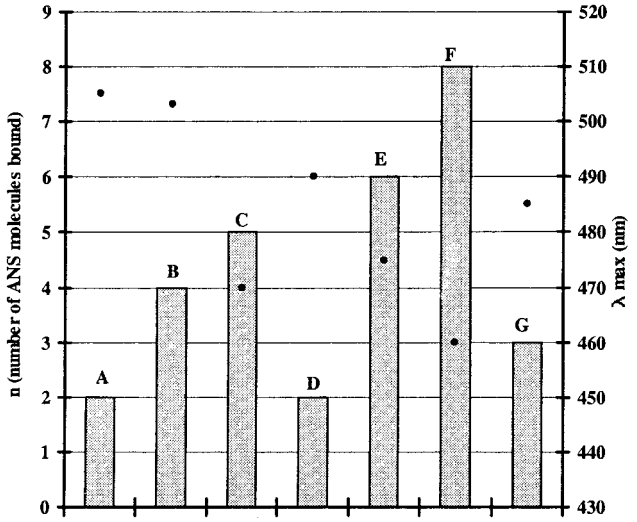

Figure 7. Results of ANS test obtained with native and modified 2S from a Scatchard plot (Scatchard et al., 1957): (A) native 2S; (B) 2S/butyric anhydride (23%); (C) 2S/butyric anhydride (93%); (D) 2S/hexanoic anhydride (24%); (E) 2S/hexanoic anhydride (60%); (F) 2S/hexanoic anhydride (89%); (G) 2S/*p*-toluenesulfonyl chloride (71%). A fixed concentration of protein (0.5 μM in 0.1 M phosphate buffer, pH 7) was titrated against ANS (253 μM in 0.1 M phosphate buffer, pH 7). Using the calibration factor ΔF_{max}, the enhancement of fluorescence observed during this titration could be converted to the number of ANS molecules bound to the protein: (■) number of ANS molecules bound to the protein; (●) maximum fluorescence emission wavelength of ANS−protein complex.

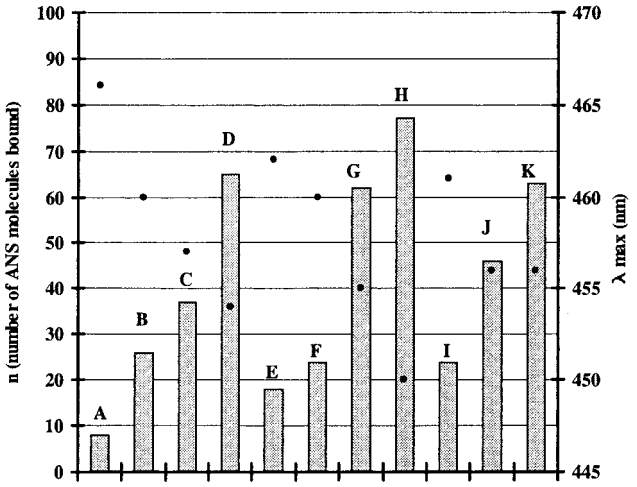

Figure 8. Results of ANS test obtained with native and modified 12S from a Scatchard plot (Scatchard et al., 1957): (A) native 12S; (B−D) 12S/butyric anhydride at 23, 39, and 61%, respectively; (E−G) 12S/hexanoic anhydride at 13, 21, 50, and 79%, respectively; (I, J) 12S/*p*-toluenesulfonyl chloride at 13 and 57%; (K) 12S/ethylbenzenesulfonyl chloride (75%). A fixed concentration of protein (0.5 μM in 0.1 M phosphate buffer, pH 7) was titrated against ANS (253 μM in 0.1 M phosphate buffer, pH 7). Using the calibration factor ΔF_{max}, the enhancement of fluorescence observed during this titration could be converted to the number of ANS molecules bound to the protein: (■) number of ANS molecules bound to the protein; (●) maximum fluorescence emission wavelength of ANS−protein complex.

Acylation did not perturb the secondary structure of 2S very much; only a slight decrease in the α-helix and an increase in the β-sheet content were noticed, leading to a structurant effect of the modification on this protein. The same effect was also observed on acylated BSA (Gerbanowski et al., 1999). Such a stabilization has

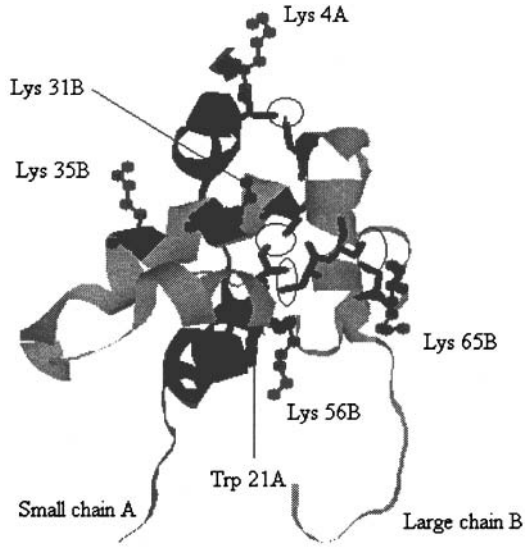

Figure 9. Three-dimensional structure of napin BnIb adapted from Rico et al. (1996): (○) disulfide bonds; (Lys) lysine residue; (Trp) tryptophan residue.

already been observed on 2S albumin in the case of acetylation or succinylation and attributed to inter- and intrachain disulfide bonds (Schwenke et al., 1988). The changes observed in sulfamidated 2S were more marked, probably due to the steric hindrance of the grafted group.

The decrease in α-helix content of 2S after modification can be explained by the location of the α-helix and lysine residues. Rico et al. (1996) studied the three-dimensional structure of a rapeseed napin isoform, BnIb. This isoform contains only five lysine residues, whereas the isoform studied here contains eight lysines, but their locations are supposed to be similar as the authors affirm that all napins and homologous 2S albumins have a similar three-dimensional structure. As shown in Figure 9, according to Rico et al. (1996), all lysine residues are exposed at the molecule surface and so are accessible to the reagent, explaining the high levels of modification reached. Furthermore, three of them are located in α-helix regions, making these latter sensitive to the modification as shown by the decrease in α-helix content observed on modified napin.

Complementary results on tertiary structure change due to modification were obtained from near-UV CD and intrinsic fluorescence. For 2S, the changes observed in near-UV CD were rather limited for short aliphatic chain grafting ($<C_4$), even for a high percentage of acylation. Conversely, for longer aliphatic (butyl or hexyl) or aromatic chain grafting, some conformational changes occurred depending on the modification percentage according to both near-UV CD and intrinsic fluorescence spectra. These tertiary structural changes are in consequence a function of the bulkiness and the number of groups grafted. According to the observed blue shift in intrinsic fluorescence of 2S acylated with long aliphatic chains, one can say that the tryptophan environment became more and more apolar as the number and the length of the aliphatic chains increased. The single tryptophan residue (Trp25 small chain) is rather exposed on the protein surface. This is confirmed by the fact that its initial maximum of fluorescence emission appeared at a wavelength close to that of exposed Trp model systems. As a consequence, the lysine residue located in its vicinity (position 28 in the small polypeptide chain) was probably one of the first residues modified. In low acylated 2S, the origin of the blue shift observed in intrinsic fluorescence is probably the result of grafting an apolar probe in the vicinity of the Trp, leading to hydrophobization of its environment. Conversely, as the other lysine residues are located rather far from Trp, the increased blue shift observed when the percentage of modification increased was probably due to conformational changes of 2S, thus inducing less exposure of Trp to the polar solvent. In fact, an increase in fluorescence with a blue shift is usually ascribed to the transfer of tryptophan groups from a polar medium to a nonpolar medium (Teale, 1960), showing a certain flexibility of the polypeptide chains in the tertiary structure of 2S.

According to the results obtained on 12S, the α-helix, β-sheet, and random coil contents of native protein are in accordance with those already published (Zirwer et al., 1985).

In contrast to 2S, the grafting of aliphatic chains resulted in a significant perturbation of the secondary structure of 12S. The Provencher results indicated a loss of α-helix and an increase in the remaining structure, indicating the destructuring effect of acylation on 12S.

Sulfamidation, like acylation, increased the remaining structure of 12S. In the same way, sulfamidation dramatically altered the secondary structure of BSA by decreasing α-helical regions and increasing the random coil structure (Gerbanowski et al., 1999). Conversely, the β-sheet content increased for 2S. The grafting of the bulky aromatic ring seems to modify the secondary structures of the proteins by perturbing the peptidic intra and interchain weak energy bonds.

For 12S, near-UV CD and intrinsic fluorescence spectra confirmed the significant perturbation of the tertiary structure after acylation or sulfamidation of the protein. Grafting long aliphatic or aromatic chains induced more perturbation than grafting short aliphatic chains. From the progressive quenching and red shift observed in the maximum fluorescence emission of acylated 12S according to the length and the number of aliphatic chains grafted, it can be deduced that the conformational changes induced by the modification lead to an exposure of Trp residues to the polar solvent (Teale, 1960). This was probably the result of an expansion of the protein (Kronman and Holmes, 1969; Chen et al., 1969), a conclusion in accordance with the greater random coil structure of modified 12S as suggested by far-UV CD spectra (Table 3). Venktesh and Prakash (1994) also observed a quenching and a red shift of the fluorescence emission as a result of increasing degrees of acetylation or succinylation of 11S proteins from sunflower seed. They interpreted these results as a progressive dissociation at lower modification levels and denaturation of the 11S at higher modification levels, resulting in exposure of the tryptophanyl groups from the interior of the protein to the aqueous solvent. In our study, ultacentrifugation experiments (data not shown) showed that no dissociation of the 12S protein occurred after grafting even for the higher degree of modification.

In conclusion, 2S exhibited a very stable conformation, only slightly changed after grafting even longer aliphatic chains; it was, however, considerably changed after sulfamidation. Conversely, the secondary and tertiary structures of 12S were dramatically perturbed

both by acylation with long aliphatic chains and by sulfamidation.

To analyze the exposure of the apolar groups grafted on the protein and to check the influence of modification on the surface hydrophobicity of 2S and 12S, the binding of an ANS probe was studied in detail. ANS is widely used to monitor conformational changes in proteins and to characterize surface exposure of hydrophobic sites (Cardamone and Puri, 1992; Butko et al., 1994; Horowitz et al., 1995). According to the results obtained by Scatchard plot, the native 2S bound only two ANS molecules. This low value is not surprising as napin is known to be a very hydrophilic protein. After acylation, the surface hydrophobicity of napin increased with the number and the length of the aliphatic chains grafted. A maximum of eight ANS binding sites was calculated for 2S highly acylated with hexanoic anhydride (90%). According to the degree of modification (90%), about seven hexanoic chains have been grafted to 2S. It can be concluded that the number of ANS binding sites is related, almost stoichiometrically, to the number of chains grafted. These latter were probably exposed to the solvent because each of them bound one ANS molecule. Moreover, this behavior is consistent with the fact that no important structural changes were noticed after acylation. Conversely, after sulfamidation of 71% of the lysine residues with *p*-toluenesulfonyl chloride, 2S bound only three ANS molecules, whereas six apolar groups have been grafted. As for the more significant variation of conformation observed for the sulfamidated 2S compared to acylated ones, this may indicate that in water solvent conditions, the aromatic chains grafted are not exposed but, by contrast, are probably orientated toward the more hydrophobic core of the protein. The difference in surface hydrophobicity between acylated and sulfamidated 2S could be due to the more hydrophobic nature of aromatic rings compared to aliphatic chains that led to groups which were probably more buried and did not interact with the ANS molecules. We have already observed such behavior with acylated and sulfamidated BSA (Gerbanowski et al., 1999).

Native 12S bound eight ANS molecules, which is close to the value found for the high-affinity class of site in the case of pea 11S protein (Guéguen, 1989). After acylation at low levels, with butyric or hexanoic anhydrides, the number of bound ANS molecules increased and was nearly equivalent, like for 2S, to the initial number of bound ANS molecules plus the number of grafted chains. Such a result indicates an exposure of the latter at the surface of the protein. In fact, for these proteins, we noticed only a quenching in intrinsic fluorescence indicating slight changes in conformation. Conversely, for highly acylated 12S, the number of bound ANS molecules was higher than the number of grafted chains. In fact, after grafting of 40 aliphatic chains (50% acylation), the protein bound 62 ANS molecules including the 8 original binding sites. The difference between 62 experimental sites and the 48 expected sites may result from a conformational change of the protein that increased the exposure of some additional hydrophobic regions which were previously buried. Confirmation of this explanation is given by the red shift observed in the intrinsic fluorescence of these proteins, which was attributed to the expansion of the protein due to the slight unfolding of the constitutive polypeptides. Besides, a blue shift was generally observed for the ANS-acylated 12S, reaching 16 nm for the 79% acylated globulin, indicating a significant hydrophobization of the sites.

For 12S sulfamidated at a low level with *p*-toluenesulfonyl chloride (13%), this phenomenon of unfolding has probably already occurred as the protein bound 24 ANS molecules, whereas only 10 aromatic chains were grafted. Conversely, when more aromatic chains were introduced (46 chains, 57% sulfamidation), the number of bound ANS molecules was surprisingly found at 46, which is lower as expected. It might signify that either the original hydrophobic sites or some grafted probes are masked due to some refolding or aggregation phenomena. As for acylation, the hydrophobization of the binding sites was very significant as shown by the blue shift of 10 nm compared to the ANS−native 12S maximum emission.

In summary, few effects of acylation on the conformation of the protein were observed for a low level of modification, whereas a limited rate of sulfamidation induced unmasking of hydrophobic regions of the oligomeric 12S protein. For a high level of grafting, a similar effect was observed for acylation. In contrast, in these conditions, sulfamidation seems to induce aggregation of the protein through hydrophobic interactions.

In conclusion, it was clearly demonstrated in this study that grafting hydrophobic chains to monomeric (2S) or hexameric (12S) proteins did not have the same effect on the structure and surface hydrophobicity of the proteins. On the monomeric protein, acylation did not induce conformational changes large enough to mask the grafted aliphatic or aromatic chains. As a consequence, the surface hydrophobicity increased drastically and the number of bound ANS is related to the number of grafted chains. Conversely, after sulfamidation, conformational changes seemed to induce masking of grafted groups, leading to a lower surface hydrophobicity than was expected. In the case of the hexameric protein, an expansion of the protein occurred, even at low levels of modification in the case of sulfamidation, leading to an exposure of numerous hydrophobic regions and, consequently, to a drastic hydrophobization of the protein surface.

Acylation and sulfamidation appear here to be very good strategies to hydrophobize efficiently the surface of rapeseed proteins and thus provide hope for a real improvement in their functional properties.

ABBREVIATIONS USED

AG_{nm}, free amino group for nonmodified protein; AG_m, free amino group for modified protein; ANS, anilinonaphthalenesulfonic acid; BCA, bicinchoninic acid; BSA, bovine serum albumin; CD, circular dichroism; OPA, *o*-phthaldialdehyde; Trp, tryptophan; UV, ultraviolet.

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