Some Physicochemical and Interfacial Properties of Native and Acetylated Legumin from Faba Beans (*Vicia faba* L.)

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The influence of acetylation on adsorption kinetics, film compression behavior (air/water), emulsifying properties (*n*-decane/water emulsions), and conformation of legumin from faba beans was studied. These measurements involved the Wilhelmy plate method, film balance, turbidity measurements, centrifugation, and size exclusion and reversed phase HPLC, UV absorption, and fluorescence probes. While distinct changes in physicochemical properties only occurred above a "critical degree of modification" (approximately 60%) e.g. dissociation and denaturation of the molecule and increased surface hydrophobicity, emulsifying property parameters linearly improved with acetylation as indicated by an increase in the emulsifying activity index of the legumin, improved coalescence stability of the emulsions, and decreased droplet size distribution and protein load of the oil droplets. The measured contact angle due to adsorption of legumin at the Wilhelmy plate distinctly influenced parameters of the Gibbs adsorption isotherms. A correlation between the critical association concentration and the emulsifying activity of the protein samples was also found.

Keywords: *Legumin; faba bean protein; conformational changes; surface adsorption; emulsion*

INTRODUCTION

Acylation has been shown to be an effective tool for improving surface functional properties of plant proteins (Franzen and Kinsella, 1976; Barber and Warthesen, 1982; Ma, 1984; Gueguen et al., 1990). Faba bean protein isolate preparations have been widely studied with regard to the influence of acylation on their surface functionality. Successive acetylation or succinylation of faba bean protein isolates results in a continuous increase in emulsifying activity and capacity of the protein as well as in emulsion stability (Rauschal et al., 1981; Schmandke et al., 1981; Muschiolik et al., 1987). However, it is very difficult to correlate changes in structural and functional properties of these proteins because they are heterogeneous molecules and contain additional surface active compounds (Krause and Buchheim, 1994). Additionally, the process of isolate preparation often leads to partial denaturation (Schwenke et al., 1993).

Very little information is available in the literature on structure−function relationship of the 11S globulin "legumin"—the main storage protein from faba beans.

This paper examines the influence of progressive acetylation on the adsorption behavior and emulsifying properties of legumin while also taking into consideration changes in physicochemical properties of this protein.

MATERIALS AND METHODS

Protein Studies. *Legumin Preparation and Acetylation.* The legumin (L) was prepared by a combined salt fractionation and isoelectric precipitation according to the method of Popello et al. (1988) as described previously (Schwenke et al., 1994). The vicilin content amounted to ≤4% as determined with SDS−polyacrylamide gel electrophoresis by Schwenke et al. (1994). Legumin acetylation was performed according to the procedure of Schwenke et al. (1989). The legumin samples were dialyzed against distilled water and freeze-dried just after preparation. A potassium phosphate buffer, pH 7.6 (0.04 M KH_2PO_4−K_2HPO_4, 0.1 M NaCl, 0.02% NaN_3, p.A., Merck, Germany), was used throughout this study. The buffer was produced with analytical grade water obtained by steam distillation of deionized water from potassium permanganate.

Table 1. Characteristics of Legumins: Degree of N-Acetylation (N-DA), Degree of O-Acetylation (O-DA), Nitrogen Content (N), and N Protein Conversion Factor (*F*)

N-DA (%)	O-DA (%)	N	*F*
0	0	15.7	5.5
34	10	16.2	5.6
60	84	15.3	5.7
84	75	15.1	5.7
96	100	15.8	5.7

Protein Concentration. The protein content of all protein solutions was determined by means of a microbiuret method (Itzhaki and Gill, 1964). For calibration, the nitrogen content of the legumins used was determined according to the Kjeldahl method, and the resulting protein content was calculated by means of experimentally determined nitrogen−protein conversion factors (Table 1).

Degree of N- and O-Acetylation. The degree of N-acetylation was determined by using the trinitrobenzenesulfonic acid (TNBS) method (Fields, 1972) modified as follows: A protein solution (0.05%) was mixed with an equal volume of a 1% solution of sodium dodecyl sulfate (SDS) and kept for 5 min in a boiling water bath. After cooling to room temperature, the solution was diluted to a final concentration of 0.05% by adding 0.2 M sodium borate buffer of pH 9.0 containing 1% SDS. After the protein solution (0.5 mL) was mixed with an equal volume of SDS-containing borate buffer and a 0.1% TNBS solution in borate buffer, the reaction with TNBS was allowed to be carried out over a time period of 1 h at 50 °C. The mixture was then cooled rapidly under running tap water, 0.5 mL of an 8% SDS solution and 0.5 mL of 1 N hydrochloric acid were added, and the absorbance was measured at 340 nm after 20 min. The degree of O-acetylation (esterification of hydroxy amino acids including tyrosine) was determined using an hydroxamic acid reaction after treatment with hydroxylamine according to the method of Habeeb and Atassi (1969) with tyrosine methyl ester used as standard. The error in the determination of the degree of acetylation was 3%.

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Surface Hydrophobicity. Surface hydrophobicity was determined from fluorescence probe measurements using *cis*-parinaric acid (CPA) and 1-anilino-8-naphthalenesulfonic acid (ANS) (analytical grade, Merck) according to the methods of Kato and Nakai (1980) and Li-Chan et al. (1985), respectively, modified as described in Schwenke et al. (1993).

UV and Fluorescence Spectroscopy. UV and fluorescence spectroscopic investigations of protein solutions (2.8×10^{-6} M in 0.05 M phosphate buffer, pH 8.0, adjusted at $I = 0.5$ with NaCl) were carried out with a Perkin-Elmer Lambda 2 dual-beam spectrophotometer (slit 2 nm, scanning rate 6 nm/s, second-derivative spectra, and smooth over 1.9 nm) and with an Aminco Bowman Series 2 (excitation 280 nm; bandpass excitation 1.0 nm, emission 0.5 nm; HV 900; scan rate 0.6 nm/s; 9 repetitions), respectively.

Liquid Chromatography. Size exclusion (SE) HPLC experiments were performed in a liquid chromatograph LC-6 (Shimadzu, Japan) on a Biosep 4000 column (Amchro). Eluent was 0.1 M Tris/HCl plus 0.3 M NaCl, pH 7.2. Reversed phase (RP) HPLC was carried out with a liquid chromatograph LC-10 (Shimadzu) on a Nucleosil C4 MPN column at 60 °C. Eluents were 0.05% trifluoroacetic acid (TFA) in water (A) and 0.05% TFA in acetonitrile (B). Gradient was from 5% B to 60% B at 1.83%/min.

Adsorption Studies. *Surface Tension and Contact Angle*. For these measurements the freeze-dried legumin samples were dissolved for 2 h by means of a magnetic stirrer at room temperature and then filtered through a cellulose acetate filter of 0.22 μm. The time-dependent surface tension $\sigma(t)$ of the air/water interface was measured with a tensiometer K12 (Krüss, Germany) based on the Wilhelmy plate method. The quartz glass vessel (inner diameter 60 mm) was first filled with buffer and equilibrated at 20 ± 0.1 °C. The surface was then sucked thoroughly with a high-grade steel capillary so as to remove any impurities present (Lunkenheimer, 1982). A standard platinum plate (Krüss) was dipped into the buffer (2 mm) and lifted up at "zero" level again. In this way, measurements were always made with a receding contact angle to minimize the contact angle (MacRitchie, 1990). Only when the surface tension of the buffer, σ_0, was 73.0 ± 0.2 mN/m over 120 min was 50−200 μL of buffer removed, and the same volume of a concentrated protein solution (0.05−5 mg/mL) was injected with a Hamilton microsyringe at the bottom of the vessel, resulting in a final bulk concentration, c_0, between 1.0×10^{-8} and 1.0×10^{-5} g/mL. After the equilibrium surface tension, σ_e, was attained, the actual bulk concentration was determined from the weight of the subphase. After the surface tension measurement of each protein solution, the plate was thoroughly rinsed with buffer to remove loosely bound protein. Subsequently, the surface tension of pure buffer, σ_ϑ, was again measured with the rinsed plate. In this way, the contact angle due to adsorbed protein at the plate was determined. With σ_0 from the beginning of the measurement and σ_ϑ measured with the "protein-contaminated" plate, the contact angle is $\cos \vartheta = \sigma_\vartheta/\sigma_0$. The slope of the corresponding curve $\vartheta = \ln(c_0)$ ($R^2 > 0.90$, $p \leq 0.001$) gave the correction factor for the measured surface tension of each protein sample used.

The following parameters were calculated from the commonly used plots (MacRitchie, 1978): The graph of the function $\log d\Pi/dt = f(\Pi)$ divided into linear parts gives the limits of further kinetics steps. The occupied interfacial area per molecule, A, the equilibrium surface pressure $\Pi_e = \sigma_0 - \sigma_e$, and the critical association concentration (CAC), the point at which the plateau value was reached, were estimated from the Gibbs adsorption isotherm $\Pi_e = f(\ln c_0)$.

Film Balance. For studying the compression behavior of the legumin samples, film pressure−area isotherms were recorded with a KSV 3000 film balance (KSV Instruments, Helsinki). Buffer was poured in the Teflon trough (450 × 150 mm), and the temperature was kept at 20.0 ± 0.2 °C with a circulating water bath. The surface temperature was permanently monitored behind the movable barrier using a digital thermometer. The buffer surface was cleaned by suction after the surface area was compressed to a minimum. This procedure was repeated until the surface pressure of the compressed area was about 0.5 mN/m after a rest period of 30 min (MacRitchie, 1985). The glass rod method according to Trurnit (1960) was used to spread 100 μL of buffer containing 0.05 mg of protein. After a spreading time of 60 min, the film was compressed from 724 to 80 cm^2 and expanded again with the barrier at constant speed of 10 mm/min. The corresponding change in film pressure was measured by the Wilhelmy method using a platinum plate. Each experiment was repeated.

Emulsion Studies. *Emulsion Formation*. Before the emulsions were formed, the legumin samples were first suspended in buffer (2 h, room temperature) and adjusted to 2 mg of protein/mL of buffer. Emulsions (total volume 5 mL) with volume fractions $\phi = 0.2$, 0.3, and 0.4 of *n*-decane (purity 99.9%, Merck) were then produced with an ultrasonic disintegrator Sonoplus HD 70 (Bandelin, Germany). The disperse phase and the continuous phase were poured into a cylindrical glass vessel (inner diameter 20 mm). The MS 73 sonotrode of the sonifier was immersed into the vessel, so that the titanium flat tip (diameter 3 mm) was in the boundary between the oil and the aqueous phase. The device, which delivers a nominal 70 W power at 20 kHz frequency, was operated for different time intervals (intervals of 0.5 s, total sonication time 120 s) at a power input of 80% (equivalent to an energy input in water of 23 W) for emulsification. The temperature of the sample during homogenization was kept between 20 and 30 °C using a circulating water bath connected with the glass vessel. The emulsion samples were transferred to 4.4 mL centrifuge tubes (Kontron) at constant filling weight (3.8 g) and stored for 24 h at room temperature.

Droplet Size d_{32}. Droplet size was determined from spectroturbidimetric measurements carried out immediately after homogenization. First aliquots (100 μL) of the emulsion were pipetted into 1 mL of a mixture containing 0.1% SDS plus 1.0% dextran and diluted once more to give an absorbance between 0.2 and 0.7. The solution was filled into a 1 cm cuvette carefully vortexed 10 times to distribute the oil droplets homogeneously. Afterward, the absorbance was measured three times—immediately after vortex and then after 1 and 3 min. The average volume/surface diameter of the droplet size distribution, d_{32} (μm), was calculated from the absorbance according to the method of Heller and Pangonis (1957) using the tables of scattering functions of Verner et al. (1976) [relative refractive index (m) as the ratio of the refractive indices from *n*-decane (1.411) to buffer (1.335) $m = 1.057$; relative scattering coefficient was set 2]. For emulsions with a negligible amount of droplets smaller than 1 μm in diameter, the calculated d_{32} values were compared with values measured with automatic imaging system CUE 3 (Olympus, Germany) as described elsewhere (Krause et al., 1996). The differences in the d_{32} values obtained from both methods were in the range of the total error of ≤10% for the emulsion parameters.

Emulsifying Activity Index and Protein Load. The emulsions were centrifuged for 24 h after homogenization at an average centrifugal force of ≈3000*g* (60 min at 20 °C). The bottom of the centrifuge tube was tapped with an injection syringe, the aqueous phase was sucked off, and the protein concentration was determined. Using the absorption data and the protein concentration of the aqueous phase, the emulsifying activity index (EAI, m^2 of oil/g of protein) according to Pearce and Kinsella (1978) and the protein load (PL, mg of protein/m^2 of dispersed phase) according to Tornberg (1978a) were calculated.

Creaming and Coalescence Stability. The creaming stability under gravity after 24 h is expressed by the volume of the separated aqueous phase. The heights of the aqueous and creamed emulsion layers were measured with a scale calibrated in milliliters. The coalescence stability was checked by using a kinetically orientated centrifugal test; i.e., the emulsions were centrifuged for a constant time, but no equilibrium state was reached. The test consisted of three runs at 10 000, 40 000, and 55 000 rpm (corresponding average radial forces: 10335*g*, 166759*g*, and 315000*g*, respectively) in a preparative ultracentrifuge Centrikon T-1170 (Kontron, Germany) with swing-out rotor TST60.4 at $T = 20.0 \pm 0.2$ °C. After each run of 30 min, the separated volumes, i.e. free oil,

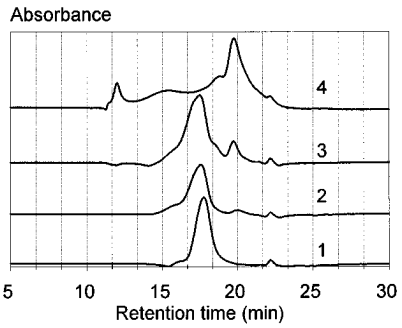

Figure 1. SE-HPLC patterns of legumins as a function of the degree of acetylation (%): (1) 0%; (2) 34%; (3) 60%; (4) 96%.

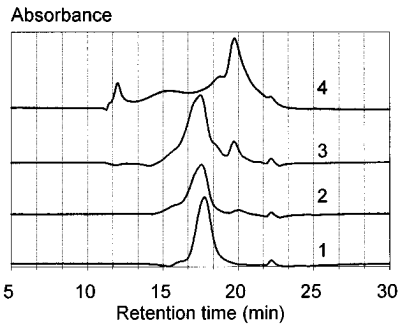

Figure 2. RP-HPLC patterns of legumins as a function of the degree of acetylation (%): (1) 0%; (2) 34%; (3) 60%; (4) 96%.

emulsion layer, and separated aqueous phase, were measured with the calibrated scale. The error in the determination of the phase volume was $\Delta V = \pm 0.04$ mL. All data represent a mean value of triplicate analyses.

Statistical Analysis. Simple linear regression analysis was used to determine the relationships between degree of acetylation and emulsifying and adsorption parameters, and the significance of the regression coefficient was checked using the *T*-test (Storm, 1986).

RESULTS

Protein Studies. *Chemical Characteristics.* Table 1 gives the nitrogen content, degree of N-acetylation, degree of N- and O-acetylation, and N-protein conversion factors. The O-acetylation markedly increased at higher degrees of N-acetylation, which contributes to the increase of the N protein conversion factor.

Liquid Chromatography. Figure 1 shows the SE-HPLC patterns of legumin at various degrees of acetylation. Unmodified legumin appeared as a main peak fraction at a retention time $R_t = 17.76$ min beside a faster running subsidiary fraction which had been previously shown to consist of aggregated legumin (Schwenke et al., 1994). In addition, a low molecular weight minor fraction ($R_t = 22.22$ min) is also present, the amount of which did not change after acetylation. The molecular mass of the unmodified legumin was determined to be 355 ± 10 kDa using calibration with molecular mass standards. This corresponds well with the value determined according to hydrodynamic methods (Plietz et al., 1984). Acetylation resulted in a successive reduction of the size of the main fraction, which disappeared at >90% acetylation. Simultaneously, the percentage of faster and slower running fractions increased. A fraction at $R_t = 20.07$ min appeared at 34% N-acetylation of legumin, which increased with further modification and amounted to about 40% of the total protein at 96% acetylation (Figure 1), the molecular mass of which was estimated to be 90 kDa and did not correspond to a subunit molecular mass but seems to reflect rather the increased Stokes radius of the expanded dissociated subunits. Similarly, faster running fractions ($R_t = 12-16$ min) may correspond to conformationally changed material with markedly increased hydrodynamic radii. Preliminary ultracentrifugation studies (results not shown) revealed the presence of a fast sedimenting component (sedimentation coefficient > 15 S) as well as a 2 S dissociation product even in exhaustively acetylated legumin, while native legumin sediments only as a 12 S component. This preliminary study confirms the results of SE-HPLC showing the formation of both products of dissociation and aggregation after legumin acetylation.

Previously published results of gel electrophoretic studies clearly revealed both the stepwise dissociation and the formation of aggregation products in legumin after high acetylation (Schwenke et al., 1991). The homologous protein of soybeans (glycinin) had been also shown to dissociate at a highly acetylated state (Barman et al., 1977).

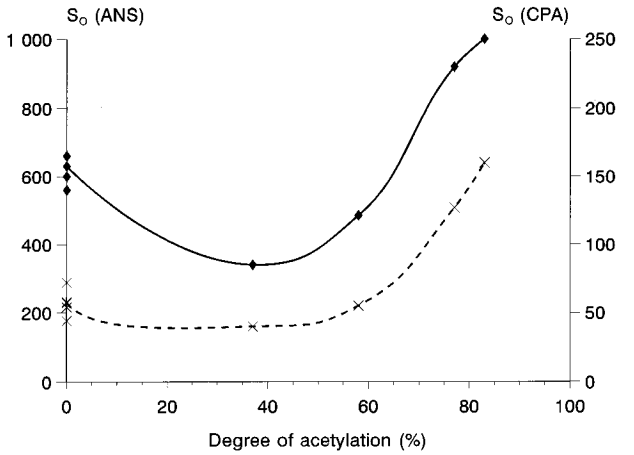

Figure 3. Surface hydrophobicity of legumin as a function of the degree of acetylation (%): (solid line) ANS method; (dashed line) CPA method.

Figure 2 shows the RP-HPLC patterns of unmodified and variously acetylated legumin. The legumin preparation was separated into two principal components (R_t = 20.78 and 21.47 min) with two subsidiary fractions (R_t = 18 and 23.13 min). Progressive acetylation resulted in a shift of the main fraction to higher retention times, thus reflecting the increased hydrophobic character of the proteins. Simultaneously, the two main components of the legumin appeared as one fraction and showed an increase in tailing with the increase in degree of modification.

Surface Hydrophobicity. The change in hydrophobicity after acetylation is confirmed by fluorescence probe techniques. Figure 3 shows the dependence of the relative hydrophobicity indices, S_0, of legumin on the degree of acetylation using CPA and ANS as fluorescence probes. It is worth noting that a sharp increase in S_0 occurred after a 60% acetylation level was attained, while only small changes in S_0 were observed at lower levels. The increased hydrophobic nature of the modified protein should be due to both the increased number of hydrophobic acetyl residues and an unfolding of the protein. These effects are reinforced by acetylation of the protein hydroxyl groups. This O-acetylation becomes increasingly important when more than 60% of the amino groups in the protein are acetylated (Table 1). Previously performed fluorescence probe measurements on faba bean protein isolates also showed a drastic increase of hydrophobicity at degrees of N-acetylation ≥60% (Schwenke et al., 1993).

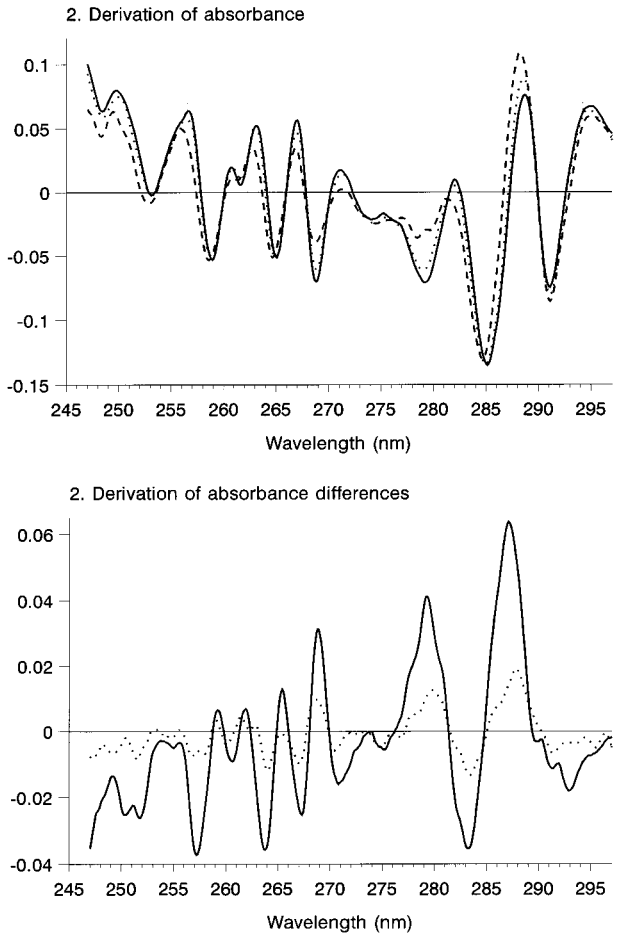

Figure 4. Second-derivative (a, top) and difference-second-derivative UV spectra (b, bottom) of acetylated legumins: (a) (solid line) legumin; (dotted line) 60% acetylation; (dashed line) 96% acetylation; (b) (dotted line) 60% acetylation; (dashed line) 96% acetylation (legumin as reference).

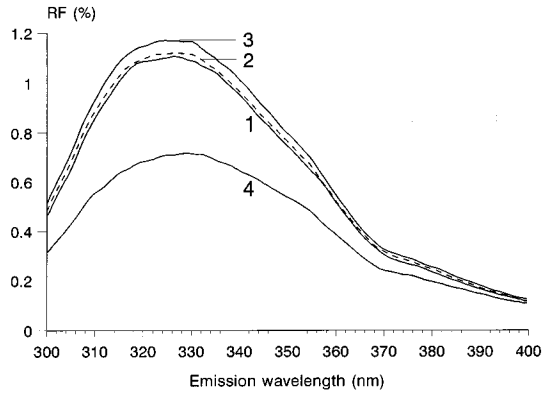

Figure 5. Fluorescence spectra of acetylated legumin: (1) 0% (legumin); (2) 34%; (3) 60%; (4) 96% acetylation.

UV and Fluorescence Spectroscopy. UV and fluorescence spectroscopic studies also revealed conformational changes at a level greater than 60% acetylation (Figures 4 and 5). The UV absorption spectrum of the unmodified protein (not shown) had a maximum at 279 nm. Acetylation led to a shift of the absorption maximum to lower wavelengths with a change of about 5 nm at 96% acetylation. This indicates a partial exposition of aromatic chromophores to the hydrophilic environment (Donovan, 1969). The second-derivative spectra of unmodified and acetylated legumin showed peaks and troughs which are characteristic of residues of the chromophores phenylalanine (250−270 nm) and tyrosine and tryptophan (>270 nm) (Figure 4a). A small blue shift but a clearly visible increase of the peak−trough difference in the region of tryptophan (285 and 288 nm) was observed at 60% acetylation, whereas the lower wavelength region remained practically unchanged. The most distinct changes occurred at 96% acetylation, where both the blue shift and the peak−trough differences in the tryptophan absorption bands further increased and clear changes in the tyrosine and phenylalanine bands were observed. The difference-second-derivative spectra showed only small changes in the 280−290 nm region after 60% acetylation, whereas all chromophores were affected after extensive acetylation (Figure 4b).

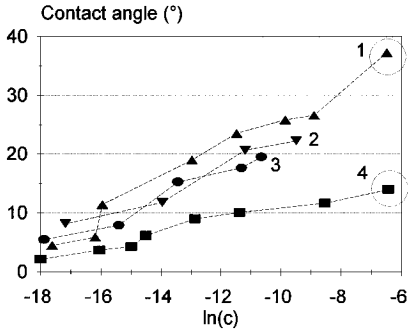

Figure 6. Calculated contact angle dependence on the initial subphase concentration and the degree of acetylation (%) (circled values were measured after 48 h of storage of the plate in a concentrated protein dispersion): (1) 0%; (2) 34%; (3) 60%; (4) 96%.

The legumin fluorescence emission spectrum had a maximum at 326 nm corresponding to a dominating tryptophan fluorescence (Figure 5). Extensive acetylation (96%) resulted in the shift of the maximum to 330 nm. The spectral red shift started to occur after 60% acetylation and is an indicator of protein unfolding (Chen et al., 1969). The protein unfolding is also reflected by fluorescence quenching appearing at a modification level >60% because, generally, denaturation of a protein is accompanied by a decrease in the fluorescence intensity caused by the changes in the environment of the tryptophan residues (Lakowicz, 1983). Moderate modification (34−60%) led to an increase in fluorescence intensity, indicating that some conformational changes, i.e. alterations in the tertiary structure (Schwenke et al., 1990), had already taken place. This was also suggested from the UV second-derivative spectra.

Adsorption Studies. *Contact Angle.* The calculated contact angle increased with increasing bulk protein concentration (Figure 6). The contact angle for the native legumin (L) was between 5° and 27°, whereas the value for the highly acetylated legumin (96%) varied between 2° and 11°.

Adsorption Kinetics. The bulk concentration of protein at which the surface pressure exceeds ca. 1 mN/m was about 2×10^{-8} g/mL and more or less independent on the degree of modification. The adsorption kinetics $\sigma(t)$ of all legumin samples are characterized by the typical induction period observed for some proteins (Tornberg, 1978b; De Feijter and Benjamins, 1987; Subirade et al., 1992), where the surface pressure $\Pi(t) = \sigma_0 - \sigma(t)$ remained below 0.5−1 mN/m followed by the diffusion controlled phase (nearly linear part in Figure 7). After this phase, the rate of change ($d\sigma/dt$) decreased and reached zero in the plateau region. Linear parts of log ($d\Pi/dt$) = $f(\Pi)$, which are the basis

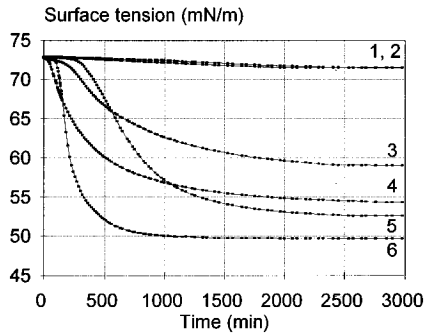

Figure 7. Surface tension σ as a function of the bulk concentration c_0 and the degree of acetylation: (1) 96% DA, $c_0 = 2.61 \times 10^{-6}$ g/mL; (2) 0% DA, $c_0 = 2.65 \times 10^{-5}$ g/mL; (3) 96% DA, $c_0 = 5.52 \times 10^{-7}$ g/mL; (4) 0% DA, $c_0 = 2.58 \times 10^{-6}$ g/mL; (5) 0% DA, $c_0 = 1.10 \times 10^{-8}$ g/mL; (6) 96% DA, $c_0 = 2.76 \times 10^{-8}$ g/mL.

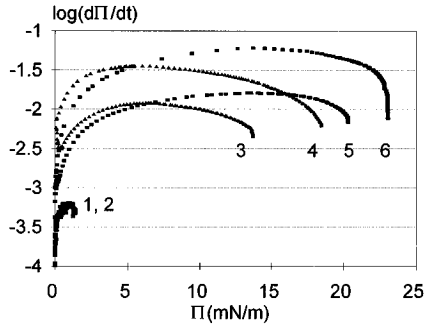

Figure 8. log (dΠ/dt) versus Π as a function of the bulk concentration c_0 and the degree of acetylation: (1) 0% DA, $c_0 = 1.10 \times 10^{-8}$ g/mL; (2) 96% DA, $c_0 = 2.76 \times 10^{-8}$ g/mL; (3) 0% DA, $c_0 = 2.58 \times 10^{-6}$ g/mL; (4) 0% DA, $c_0 = 2.65 \times 10^{-5}$ g/mL; (5) 96% DA, $c_0 = 5.52 \times 10^{-7}$ g/mL; (6) 96% DA, $c_0 = 2.61 \times 10^{-6}$ g/mL.

Table 2. Critical Association Concentration (CAC), Plateau Value of the Surface Pressure (Π_e), and Occupied Area (A) with and without Contact Angle Correction as a Function of the Degree of Acetylation (DA) Calculated from the Gibbs Isotherms

DA (%)	CAC (g/mL)	CAC_{ac} (g/mL)	Π_e (mN/m)	$\Pi_{e,ac}$ (mN/m)	A (nm^2)	A_{ac} (nm^2)
0	1.25×10^{-4}	7.66×10^{-5}	23.56	16.60	1.73	2.47
34	5.11×10^{-6}	5.39×10^{-6}	24.27	21.44	1.00	1.08
60	1.71×10^{-6}	1.51×10^{-6}	24.61	22.52	0.76	0.80
96	1.05×10^{-6}	8.96×10^{-7}	23.67	23.07	0.73	0.72

for calculating further adsorption barriers (MacRitchie, 1978), could not be detected (Figure 8).

Adsorption Isotherms. The calculated parameters of the Gibbs adsorption isotherms (not shown), i.e. equilibrium pressure versus initial bulk concentration of protein, consisting of at least 20 different bulk concentrations are summarized in Table 2. The critical association concentration (CAC) decreased with increasing degree of acetylation, whereby the highly acetylated legumin (96%) had the lowest CAC (1.05×10^{-6} g/mL). The plateau values also displayed a distinct dependence on the degree of acetylation (unmodified legumin 16.60 mN/mn, 96% acetylated protein 23.07 mN/m).

Compression Isotherms. The $\Pi-A$ compression isotherms are shown in Figure 9. The film of the unmodified legumin could be compressed to 50% of its initial area before the film pressure Π changed significantly, while Π of the highly acetylated protein film changed at low percentages of compression. The films did not show any sign of collapse at the highest values of Π_e (Π_e = 31 mN/m for legumin; Π_e = 41 mN/m for 96% acetylated legumin). The surface concentrations (mg/m^2) calculated from the $\Pi-A$ isotherms at moderate film pressures were 2.00 mg/m^2 for the unmodified legumin and 1.04 mg/m^2 for the 96% acetylated legumin.

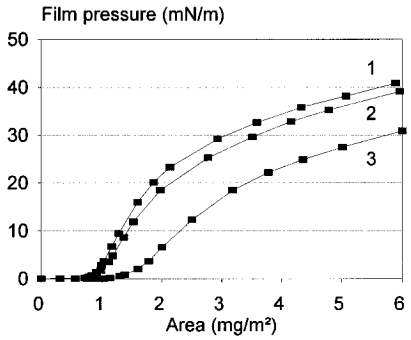

Figure 9. $\Pi-A$ isotherms as a function of the degree of acetylation (%) at a protein deposition of 0.05 mg: (1) 96%; (2) 60%; (3) 0%.

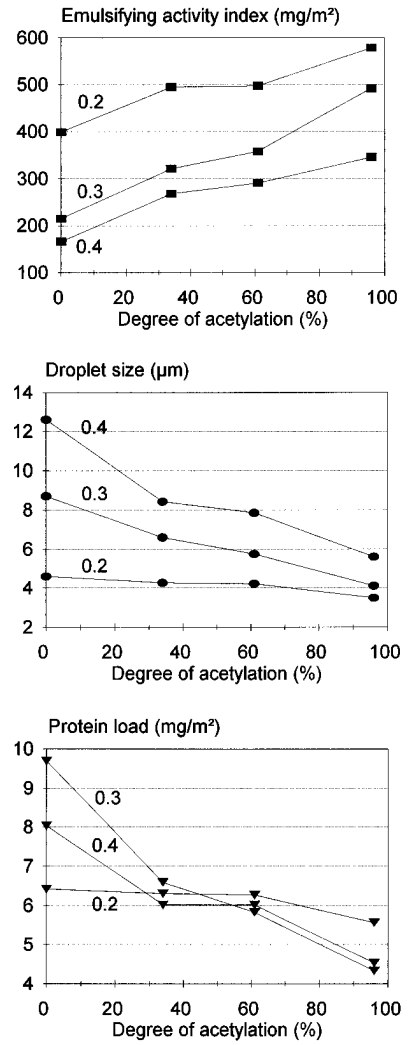

Figure 10. EAI (a, top), droplet size (b, middle), and protein load (c, bottom) vs degree of acetylation at oil fractions of ϕ = 0.2, 0.3, and 0.4 (2 mg of protein/mL of buffer).

Emulsion Studies. *Emulsifying Properties.* Figure 10 illustrates changes in emulsifying properties of the legumin with acetylation for different oil fractions ϕ = 0.2, 0.3, and 0.4. Table 3 shows the corresponding correlation coefficients, R^2, of the linear regression between degree of acetylation and EAI, d_{32}, and PL.

For ϕ = 0.2 the EAI increased significantly with increasing degree of acetylation, but the d_{32} values and

Table 3. Correlation Coefficient R^2 (Level of Significance $p \leq 0.001$) between the Degree of Acetylation (DA) as well as the Critical Association Concentration (CAC) and the Emulsifying Parameters EAI, d_{32}, and PL at Different Volume Fractions of Disperse Phase ϕ ($n = 12$, *$p \leq 0.01$)

	$\phi = 0.2$			$\phi = 0.3$			$\phi = 0.4$		
	EAI (g/m^2)	d_{32} (μm)	PL (mg/m^2)	EAI (g/m^2)	d_{32} (μm)	PL (mg/m^2)	EAI (g/m^2)	d_{32} (μm)	PL (g/m^2)
DA (%)	0.7037	0.5306*	0.5420*	0.9219	0.9219	0.9199	0.8954	0.8448	0.8202
CAC_{ac} (g/mL)	0.5719	0.2671*	0.1828*	0.5911	0.7301	0.8506	0.8074	0.7786	0.7095

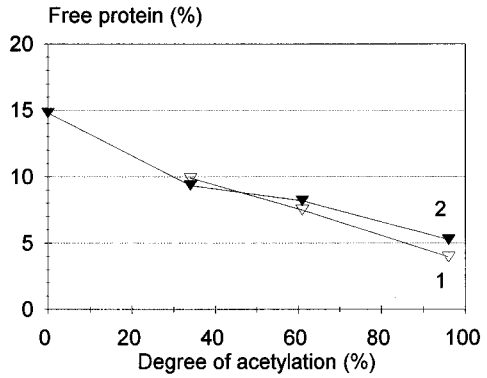

Figure 11. Protein content of the aqueous phase (%) related to the total amount of protein in the emulsion ($\Phi = 0.4$, 2 mg of protein/mL of buffer) after the centrifugal test (1) and after the determination of free protein (2).

PL remained almost constant within the ranges 3.5−4.6 μm and 5.23−6.47 mg/m^2, respectively. At $\phi = 0.3$ and 0.4 all parameters distinctly changed with progressive acetylation. Now, the calculated protein load decreased with progressive acetylation from 9.7 (legumin) to 4.5 mg/m^2 (96% acetylation). Thus, PL is in the common range for protein-stabilized emulsions (Walstra, 1988) and points to aggregated multilayers (Krog et al., 1989). The droplet size decreased from about 13 to 4 μm, and the EAI is lowered with increasing oil fraction.

Creaming and Centrifugal Stability. After 24 h of storage, creaming was observed only in emulsions with $\phi = 0.2$ and 0.3, with no dependence on the degree of acetylation. The coalescence stability increased with acetylation, but no quantitative results are possible because of the nonequilibrium conditions of the test. Emulsions stabilized with unmodified legumin coalesced at 55 000 rpm for $\phi = 0.3$ and even at 40 000 rpm for $\phi = 0.4$, whereas all other emulsion were stable under the chosen conditions (not shown). After centrifugation at 20 000 rpm, a transparent layer between the opaque layer and the oil was observed in all emulsions. Under gravitational force, drainage of the continuous phase between the oil droplets is accompanied by a decreasing thickness and a change of the optical properties of the interfacial film as discussed elsewhere (Krause et al., 1995). The small amount of protein in the separated aqueous phase of legumin-stabilized emulsions (ca. 15%) was also reduced with increasing degree of acetylation (Figure 11). The small dependence on the centrifugal stress is in the limit of the experimental error.

Relationship between Emulsifying and Adsorption Behavior. The linear regression analysis between the contact-angle-corrected CAC as independent variable and the emulsifying parameters, EAI, d_{32}, and PL are also summarized in Table 3. While at $\phi = 0.2$ no high correlation was found, at $\phi = 0.3$ and 0.4 the R^2 values were found to be highly significant ($p \leq 0.001$). By contrast, the correlation between the CAC and Π_e was not significant (not shown).

DISCUSSION

The properties of acylated 11S proteins that improve its "interfacial functionality" (e.g. emulsifying activity, adsorption behavior) are the profound changes in its conformation. Partial unfolding of the proteins to expose hydrophobic groups results in improved emulsifying properties (Voutsinas et al., 1983), decreasing surface tension, and increasing film pressure (Subirade et al., 1992). At a fixed pH, the higher net negative charge of acetylated proteins caused by blocking of positively charged groups presumably enhances electrostatic repulsion between protein-coated oil droplets, leading to a greater emulsion stability (Kinsella and Whitehead, 1988). The significant correlation between the effective hydrophobicity and the surface-active properties of proteins first reported by Kato and Nakai (1980) has been proved for different plant proteins, e.g. soy and sunflower, etc. (Nakai et al., 1980).

Both UV absorption and fluorescence investigations revealed only small conformational changes of legumin at moderate acetylation up to a degree of 60% modification, while marked changes of the protein conformation were indicated at extensive modification. This was underscored by the course of surface hydrophobicity showing a drastic increase at a degree of modification higher than 60%. These results coincide with previous findings that the unfolding of 11S globulins due to succinylation takes place after passing a distinct level of acylation defined as the "critical degree of modification", where also a marked O-acylation begins (Schwenke et al., 1992). Succinylation of various 11S globulins leads to a complete dissociation into smaller subunits (Schwenke et al., 1992). In contrast, acetylation of the legumin samples used resulted in both products of dissociation and aggregation as shown by gel filtration analysis. A high percentage of "polymerized" products besides "dissociated" subunits in extensively acetylated soybean glycinin has also been reported by Yamauchi et al. (1979).

Changes in physicochemical properties of the legumin of faba beans occurred at a critical level of modification, but both adsorption and emulsification properties depend more or less linearly on the degree of acetylation. Already at the lowest degree of modification (34%), acetylated legumins were shown to be superior to the unmodified protein in all determined parameters of the interfacial activity. The main reason for this difference between alteration in structural and interfacial properties of the unmodified and partial acetylated legumin could be the very different environment of the molecule in solution and at interfaces. The strong interactions of the water molecules around the protein molecule, which oppose conformational changes (Hvidt and Westh, 1992), are drastically reduced at interfaces. Thus, structural changes of adsorbed proteins can easily occur and thus lead to measurable changes in macroscopic properties, such as surface pressure or emulsion stability.

The improved emulsifying properties of the acetylated legumins can be explained by the proved exposition of

hydrophobic groups at the surface of the molecule due to partial dissociation and the decay of the main 11S component in favor of a 2S component after acetylation, which renders a higher degree of unfolding at the interface and, consequently, a higher amount of hydrophobic contacts. Thus, the degree of unfolding mainly seems to determine the coalescence stability as previously discussed by Tornberg and Ediriweera (1988). Results presented here are corroborated by analytical ultracentrifugal investigations on legumin-stabilized emulsions by Seifert and Schwenke (1995). They found a 3-fold increase in the coalescence pressure of emulsions stabilized by the same highly acetylated legumin over emulsions with native legumin.

Despite the relatively large d_{32} values found between 4 and 13 μm, which imply rapid creaming of emulsion under gravity (Tornberg, 1980), no creaming was observed at an oil fraction of 0.4. This could be due to a stabilization of the emulsion structure by coagulate formation as discussed for protein-isolate-stabilized emulsions by Seifert et al. (1990). Additionally, the amount of protein in the separated aqueous phase shows no significant dependence on the centrifugal force, which also points to protein coagulates in the emulsions.

The same structural alterations of the molecule that are important for emulsification are responsible for the decrease in surface tension of the A/W interface with time and protein concentration. After the contact angle correction, the plateau values of the adsorption isotherms, Π_e, which indicate the largest reduction in surface tension, increase with acetylation. Unmodified legumin having the lowest surface hydrophobicity shows also the highest affinity to the hydrophilic surface of the platinum plate, which results in its having the highest contact angle. With an increasing degree of acetylation, i.e. more exposed hydrophobic groups at the molecule surface, the affinity to the hydrophilic plate lessens.

The proteins produced the well-known "high affinity" adsorption isotherms, i.e. high adsorption even at low subphase concentration and a plateau over a large concentration range (De Feijter et al., 1987). Below the plateau region, the equilibrium surface pressure is a linear function of the logarithm of the bulk concentration, c_0, and can be described by the Gibbs equation: $d\Pi/d(\ln c_0) = kT/A$, where A defines the occupied area per adsorbed molecule segment, k is Boltzmann's constant, and T is the absolute temperature. The commonly used two parts of an adsorption isotherm could be constructed with sufficient mathematical accuracy ($R^2 \geq 0.95$, $p \leq 0.001$). The linearly rising and the constant parts of the isotherm are separated by an inflection point, the critical association concentration (CAC). This corresponds to a critical micelle concentration (cmc) for low molecular weight surfactants. Sometimes the CAC is referred to as the point at which adsorbing globular molecules are prevented from unfolding by already adsorbed molecules in the primary layer (Graham and Phillips, 1979). Thus, the number of molecules adsorbed at the interface can also depend on their molecular size. Progressive acetylation leads to an increasing amount of a dissociated "subunit". Because of its smaller size, more of these subunits can pack into the primary adsorbed layer than into the corresponding layer of the native legumin. The result is a lower surface tension (Figure 7) and a higher film pressure (Figure 10) for the same initial protein concentration for acetylated legumins than for the unmodified legumin.

The model of adsorption barriers (MacRitchie and Alexander, 1963), represented by linear parts of the curve $\log(d\Pi/dt) = f(\Pi)$, was not applicable, as also reported for lysozyme (De Feitjer and Benjamins, 1987), β-lactoglobulin (MacRitchie, 1989), and bovine serum albumin (Paulsson and Dejemek, 1992), which means that Π is not a linear function of the surface concentration.

Information is limited in the literature on the adsorption behavior of homologous proteins. Dagorn-Scaviner et al. (1986) and Subirade et al. (1992) studied the adsorption of native pea legumin at the air/water surface at pH 8 and found a CAC of 1.85×10^{-5} g/mL, an occupied area of 1.06 nm^2, and a plateau value Π_e of 24.5 mN/m. Gueguen et al. (1990) reported for the 12S protein of rapeseed at pH 9.2 an area of 0.81 nm^2. The contact-angle-corrected values for the legumin from faba beans, however, differ considerably (CAC = 7.66×10^{-5} g/mL, A = 2.47 nm^2, and Π_e = 16.60 mN/m, respectively).

Subirade et al. (1992) observed that the CAC values of succinylated pea legumins were lower than those for the native protein at constant pH of 8, while the 60% succinylated sample showed the lowest CAC (0.83×10^{-6} g/mL) and the 95% succinylated legumin gave a value of 3.9×10^{-6} g/mL. The Π_e values were also reduced (23.1 and 22.2 mN/m for 60% and 95% succinylation, respectively), but the calculated areas were 0.93 and 1.8 nm^2 for the 60% and 95% modified samples, respectively. It was concluded that the proved dissociation of succinylated pea legumin lowers the plateau value and that exhaustively succinylated protein spreads at the interface due to repulsive effects of the negative charge, thus increasing the occupied interfacial area.

Succinylation transforms positively charged amino groups into negatively charged ones, whereas acetylation only neutralizes positively charged amino groups (Means and Feeney, 1971). This could lead to smaller electrostatic repulsion, and, consequently, to smaller spreading of the highly acetylated sample at the interface, which results in lower interfacial areas for the acetylated legumins from faba beans. In addition, the formation of a contact angle which possibly influences the measured surface tension was not considered in the above-mentioned studies. Thus, the contact angle correction carried out in our study could be a further reason for the distinct differences in interfacial properties between the globulins.

The correlation between adsorption and emulsifying activity of the proteins was only found to be significant in the limited range of volume fraction ϕ of 0.3−0.4. When ϕ is out of this range, existing differences in emulsifying properties of the proteins are hardly measurable. The first case obviously exists for $\phi = 0.2$, and for the latter, coalescence can only occur immediately after emulsification.

The missing correlation between Π_e and emulsifying activity index supports the view (Halling, 1981; Krog et al., 1989) that the reduction of the interfacial tension by proteins may not play such an important role in the formation of emulsions as often believed.

CONCLUSIONS

Studies of the change in interfacial activity of legumin from faba bean have shown that acetylation is a powerful tool to improve the surface activity of proteins.

Structural alterations (e.g. increasing hydrophobicity, dissociation of the globular molecule) lead to a marked decrease of the interfacial tension at low bulk concentrations ("CAC shift"), an increasing emulsifying activity of the protein, and coalescence stability of corresponding emulsions. The specific structural changes caused by acetylation are reflected by gradual changes in parameters of the surface activity at the planar surface as well as in emulsions. The correlation between the degree of acetylation and both adsorption behavior and emulsifying activity of the samples was found to be significant. Additionally, under particular emulsifying conditions, the relation between the affinity of the protein to the A/W interface and its emulsifying activity was also significant. A simple check of the contact angle gives valuable information on the alteration in surface tension caused by a changing affinity of the protein to the Wilhelmy plate, which could even provide new aspects in the discussion on relationships between changes in conformation and interfacial adsorption of proteins.

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