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- Flament, I. Some Recent Aspects of the Chemistry of Naturally Occurring Pyrazines. In *The Quality of Foods and Beverages*; Charalambous, G., Inglett, G., Eds.; Academic Press: New York, 1981; Vol. 1, pp 42-48.
- Hartman, G. J.; Carlin, J. T.; Hwang, S. S.; Bao, Y.; Tang, J.; Ho, C.-T. Identification of 3,5-Diisobutyl-1,2,4-trithiolane and 2-Isobutyl-3,5-diisopropylpyridine in Fried Chicken Flavor. *J. Food Sci.* 1984, 49, 1398, 1440.
- Henderson, S. K.; Nawar, W. W. Thermal Interaction of Linoleic Acid and Its Esters with Valine. *JAOCS, J. Am. Oil Chem. Soc.* 1981, 58, 632-635.
- Huang, T. C.; Bruechert, L. J.; Hartman, T. G.; Rosen, R. T.; Ho, C.-T. Effect of Lipids and Carbohydrates on Thermal Generation of Volatiles from Commercial Zein. *J. Agric. Food Chem.* 1987, 35, 985-990.
- Hwang, S. S.; Carlin, J. T.; Bao, Y.; Hartman, G. J.; Ho, C.-T. Characterization of Volatile Compounds Generated from the Reactions of Aldehydes with Ammonium Sulfide. *J. Agric. Food Chem.* 1986, 34, 538-542.
- Maga, J. A. Pyrazines in Foods: an Update. *CRC Crit. Rev. Food Sci. Nutri.* 1982, 21, 1-48.
- Majlet, P.; Erdos, Z.; Takacs, J. Calculation and Application of Retention Indices in Programmed Temperature Gas Chromatography. *J. Chromatogr.* 1974, 91, 89-110.
- Rizzi, G. P. Formation of Pyrazines from Acyloin Precursors under Mild Conditions. *J. Agric. Food Chem.* 1988, 36, 349-352.
- Shibamoto, T.; Bernhard, R. A. Investigation of Pyrazine Formation Pathways in Sugar-Ammonia Model Systems. *J. Agric. Food Chem.* 1977, 25, 609-614.
- Tang, J.; Jin, Q. Z.; Shen, G. H.; Ho, C. T.; Chang, S. S. Isolation and Identification of Volatile Compounds from Fried Chicken. *J. Agric. Food Chem.* 1983, 31, 1287-1292.

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Effect of Succinylation on Some Physicochemical and Functional Properties of the 12S Storage Protein from Rapeseed (*Brassica napus* L.)

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The physicochemical and functional properties of the native and succinylated 12S storage protein from rapeseed have been studied and found to depend on the degree of modification. Ultracentrifugation and isoelectric focusing were used for the characterization of the protein samples. The functionality was studied through adsorption kinetics at air/water interfaces and foaming and emulsifying properties. Succinylation results in a significant change of the interface adsorption kinetics of the native rapeseed globulin and in an improvement of foam capacity and stability as well as emulsion stability. These effects, greatly influenced by the level of succinylation, are discussed as being related to the dissociation of the oligomeric 12S globulin into subunits, to the unfolding of these subunits, and to the charge variations, depending on the degree of modification.

The 12S globulin represents a main storage protein in the seeds of *Brassica* species (Bhatty et al., 1968; Goding et al., 1970). It shows the typical properties of the oligomeric 11-12S plant storage proteins: a molecular weight around 300 000 (Schwenke et al., 1980), a quaternary structure composed of six subunits (Plietz et al., 1983; Schwenke et al., 1983a), a low content of α -helix and a high content of β -sheet (Schwenke et al., 1975; Zirwer et al., 1985), and two types of polypeptide chains with molecular weights around 30 000 and 20 000 forming the subunits of MW 50 000 (Schwenke et al., 1983a; Dalgalarondo et al., 1986).

It has been shown by ultracentrifugation, viscometry, and CD spectroscopy that the spatial structure of the protein can be broken step-by-step by succinylation

(Schwenke et al., 1986). This kind of modification, mainly performed for improving the functional properties of food proteins (Kinsella, 1976), has been applied to protein isolates from soybean (Franzen and Kinsella, 1976), peanut (Beuchat, 1977), sunflower seed (Canella et al., 1979; Schwenke and Rauschal, 1983), cottonseed (Choi et al., 1981), faba bean (Rauschal et al., 1981; Schwenke et al., 1983b; Prah and Schwenke, 1986), and pea (Jonhson and Brekke, 1983).

A first investigation of functional properties of the succinylated rapeseed globulin was described by Nitecka and Schwenke (1986). A certain dependence of functional properties on the kind and level of modifications was found, but the structure-function relationships were not very often studied. It was the aim of the present work to investigate this relationship, expecting that the modification of size and charge induced by succinylation would influence the surface behaviors of the rapeseed globulin. It has been shown indeed by Graham and Phillips (Gra-

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ham and Phillips, 1976, 1979a,b; Phillips, 1977, 1981) and Tornberg (Tornberg, 1978a,b; Tornberg et al., 1982) on model proteins as well as by Dagorn-Scaviner et al. (1986, 1987) on oligomeric plant seed proteins that the interfacial adsorption of proteins and finally their foaming and emulsifying properties were closely related to their molecular characteristics. Consequently, the adsorption kinetics at the air/water interface of the various succinylated samples were also carried out in the present studies to provide some of the basic information needed to explain as much as possible their emulsifying and foaming properties.

MATERIALS AND METHODS

Preparation of the 12S Globulin. The protein was isolated from dehulled and diethyl ether extracted rapeseed flour (prepared from *Brassica napus*, var. Sollux), by extraction with standard phosphate buffer (0.05 M phosphate buffer, pH 8.0, according to Sørensen, brought to an ionic strength of 1.0 with sodium chloride), precipitation by 90% saturation with ammonium sulfate, redissolution at 20% ammonium sulfate saturation, and chromatographic purification on a Sephadex G-200 column as described previously (Raab and Schwenke, 1984).

Succinylation of the Protein. The succinylation was carried out by addition of dry succinic anhydride (quality reagent, purchased from VEB Laborchemie Apolda, GDR) with stirring to a 1–2% solution of the protein in phosphate buffer (0.05 M phosphate, pH 8.0, according to Sørensen, brought to an ionic strength of 0.5 with sodium chloride). The pH was kept constant during the reaction by addition of 1 M NaOH. After termination of the reaction, the solution was dialyzed exhaustively against distilled water to remove salt and succinic anhydride and the protein freeze-dried. The degree of succinylation was estimated by determination of the free amino groups using the TNBS (trinitrobenzenesulfonic acid) method (Habeeb, 1966) with the following modifications. The reaction was carried out in 0.05 M Na_2HPO_4 /0.05 M NaCl, pH 9.2, containing 0.29% SDS at the reaction temperature of 60 °C and the incubation time of 2 h according to Barber and Warthesen (1982). The remaining free amino groups were estimated by the determination of the available lysine (Booth, 1971).

Isoelectric Focusing. Isoelectric focusing was performed on 10 mg of protein in a linear sucrose gradient on an LKB 8101 column as described previously (Schwenke et al., 1981). Ampholine (2%, pH 4–8) were used as carrier ampholyte. After focusing, 1.5-mL fractions were collected at a flow rate of 1.75 mL/min. The pH values were measured at 4–5 °C, and the absorbance was measured at 278 nm.

Ultracentrifugation. The ultracentrifugation was performed in a 5–20% sucrose density gradient according to Martin and Ames (1961) in a Beckman L5 65B type ultracentrifuge equipped with a swing-out rotor (Type SW40TI) for 21 h at 35 000 rpm (218 000g). For calibration, catalase (11.2 S), γ -globulin (7.0 S), BSA (4.4 S), and lysozyme (1.9 S) were used. The proteins were dissolved in 0.05 M Na_2HPO_4 /0.05 M NaCl, pH 9.2, and 0.5 mL of the solution at a concentration of 5 mg/mL was brought onto the top of the sucrose gradient.

Determination of Adsorption Kinetics at Air/Water Interfaces. The surface tension γ was measured at 20 °C by the Wilhelmy plate technique using a Prolabo tensiometer. The γ - t curves were recorded until a constant value of γ was reached. The tensiometer trough (approximate diameter 68 mm and height 37 mm) was filled with 25 mL of 0.5 M Na_2HPO_4 /0.05 M NaCl, pH 9.2. Protein solutions (100 μL , 1–10 mg/mL) were injected, resulting in a final concentration between 0.004 and 0.04 mg/mL. The protein sample in the lower phase was mixed by a moderate and short (20-s) magnetic stirring.

In order to determine the various parameters controlling the successive kinetic steps of the interfacial adsorption, the commonly used plots (Ward and Tordai, 1946, 1952; Graham, 1976; Tornberg, 1978a; Tornberg et al., 1982) $\pi = f(t^{1/2})$, $\ln(d\pi/dt) = f(\pi)$, and $\ln(1 - \pi/\pi_e) = f(t)$ were established on a computer. π and π_e are, respectively, the interfacial surface pressures at the time value t and at equilibrium. The interfacial pressure π

Figure 1. Foaming properties: (a, top) experimental curve, conductivity $f(t)$; (b, bottom) kinetic interpretation, $\ln(C - C'/C_1) = f(t)$.

plotted vs $t^{1/2}$ showed a linear region, giving the limits of the diffusion-controlled step (Tornberg, 1978a).

As previously described (Dagorn-Scaviner, 1986), the $\ln(d\pi/dt)$ vs π curve was divided by regression analysis into successive linear parts, each of them corresponding to a kinetic step. According to the equation given by Tornberg (1978a), $\ln(d\pi/dt) = k' - \pi\Delta A/kT$ (k' = constant, k = Boltzmann's constant, T = temperature), ΔA can be calculated from the slope of the corresponding linear sections. ΔA is defined as the mean surface area that one active segment of the protein needs to adsorb at the interface and is considered as a measure of the energy barrier to adsorption. The rate constant $R = 1/\tau$ (τ = relaxation time) of each kinetic step can be obtained from the first-order equation $\ln(1 - \pi/\pi_e) = -t/\tau$ (Graham and Phillips, 1979a); it is given by the slope of the corresponding linear segment of the graph $\ln(1 - \pi/\pi_e)$ vs time (t).

Foaming Properties. Foaming capacity and stability were determined as described by Dagorn-Scaviner (1986). The foaming was performed by bubbling a nitrogen stream through a fritted glass at the bottom of a column containing the protein solution (1 mg of protein/mL in 0.05 M Na_2HPO_4 /0.05 M NaCl, pH 9.2). The column was equipped with a conductimeter for measuring the foam capacity, which was defined to correspond to the initial conductivity of the foam (C_1 , $\mu\text{S}/\text{cm}$), according to Kato et al. (1983).

Two parameters were chosen for the determination of the foam stability. The first corresponded to the time ($t_{1/2}$) at which the conductivity (C) reached half of its initial value ($C_1/2$). The second was the foam stability index (FSI) defined according to Kato et al. (1983) as $\text{FSI} = C_0\Delta t/\Delta C$ (min), where C_0 is the conductivity at zero time obtained by extrapolation of the linear part of the C vs t plot, $\Delta C/\Delta t$ being the slope of this linear region (Figure 1a). The foam stability index, more related to the last part of the conductivity curve, especially describes the coalescence phenomena that occurred at the end of the foam-breaking process.

To evaluate the rate constants of foam breaking, the method of Dagorn-Scaviner (1986) was used. It was assumed, accord-

ing to Mita et al. (1978), that this phenomenon is ruled by first-order reactions; then drainage and coalescence between bubbles will be expressed by the change of the volume fraction of the liquid phase (Φ) in the foam, as

$$\Phi = \Phi_i e^{-\alpha t} \quad (1)$$

where Φ_i = initial volume of Φ at $t = 0$ and t = time.

Bruggeman's equation $C/C_s = \Phi^{3/2}$, where C and C_s are, respectively, the conductivities of the foam and of the protein solution, led to

$$\ln(C/C_i) = -\alpha t \quad (2)$$

where C and C_i are, respectively, the values of the foam conductivity for time values t and $t = 0$. α is the rate constant.

According to this equation, and plotting $\ln(C/C_i)$ vs t , a curve composed of linear segments was expected, but unfortunately it was not so. The explanation was the meaning of the experimental variation of C , which is due to both drainage and coalescence. These two phenomena, which are related to the foam breaking, should be ruled by different kinetics. Therefore, eq 2 was applied to drainage only by using the corrected conductivity ($C - C'$) instead of the experimental one (C). It led to

$$\ln[(C - C')/C_i] = -\alpha t \quad (3)$$

The correction term C' represents the contribution of coalescence to the conductivity values C . Assuming that the foam-breaking process is mainly due to coalescence in the last part of the curve, C' was calculated by extrapolation to the whole curve of the linear part of the experimental curve, which was previously used for the FSI determination. Under these conditions, the plot $\ln[(C - C')/C_i]$ vs time led to linear segments. According to eq 3, their slopes are the drainage rate constants (α). Three successive steps were generally observed (Figure 1b).

Emulsion Stability. Emulsion stability was determined by following the flocculation-creaming, using protein solutions (1 mg of protein/mL of 0.05 M Na_2HPO_4 , 0.05 M NaCl, pH 9.2) in a water/dodecane system as described previously (Dagorn-Scaviner, 1986; Dagorn-Scaviner et al., 1987). *n*-Dodecane from Sigma (99%) was used.

The emulsion was prepared by homogenizing 18 mL of protein solution with 6 mL of dodecane in a Polytron (PT 10/35) homogenizer (30 s at 20 000 rpm). The emulsion formed was transferred into a graduated 10-mL vessel with a syringe. The process of separation of the water phase and the creamed phase was followed over 60 min at 25 °C. The volume of the separated water phase (V) was plotted vs time (t). Assuming an equilibrium of the phase separation in 24 h, the water-phase equilibrium volume (V_e) was determined after this time. According to Dagorn-Scaviner et al. (1987), three linear regions were determined from the $\ln[V_e/(V_e - V)]$ vs t plot, corresponding to successive first-order kinetics of emulsion breaking. Each kinetic step is characterized by its rate constant β and its duration t . The equilibrium state ($t \rightarrow \infty$) of the phase separation is defined by V_e or the limit Φ_e of the volume fraction of the oil in the concentrated emulsion forming the upper creamed phase after 24 h. Φ_e is obtained from

$$\Phi_e = \frac{\text{init vol of oil (2.5 mL)}}{\text{total vol of emulsion (10 mL)} - V_e}$$

assuming that all the oil fraction sedimented in the creamed phase.

RESULTS

Physicochemical Properties. Ultracentrifugation of the unmodified globulin in the sucrose density gradient gave one peak with a sedimentation coefficient of 12S. Succinylation induced a stepwise dissociation into 7S and 3S components (Figure 2). At a degree of succinylation $\geq 66\%$, only the 3S component was detectable. At the level of 65–70%, previously characterized as a critical level of modification for 11S globulins from plant seeds (Schwenke et al., 1985), the distribution of dissociation products appeared to be the broadest.

Figure 2. Percentage of the dissociation products induced by succinylation.

Succinylation results in a shift of the isoelectric point of the native protein from 7.1 (uncorrected) to lower pH values (Figure 3a–d). The most pronounced change was observed after a modification of 29% of the amino groups, giving products with isoelectric points in the range of pH 4.23–5.25 and a maximum at pH 4.70 (Figure 3b). The critical step of 65% modification resulted in fractions in the range pH 4.15–4.80 (maximum at pH 4.55) (Figure 3c). At an extreme degree of succinylation (93%) a sharp fraction with a shoulder appeared focusing, respectively, at pH 4.20 and 4.50 (Figure 3d). In fact, this fraction consists of three sharply separated zones in the isoelectric focusing column corresponding to modified subunits.

Functional Properties. Kinetics of Adsorption and Pressure Isotherm at the Air/Water Interface. The adsorption of the protein samples at the air/water interface was followed by either the pressure isotherms (Figure 4) or the kinetics of adsorption (Figure 5). The pressure isotherm was only established for the native and the highly succinylated (83%) samples, because of the small amounts of the other samples available. It was observed that the same plateau value was reached (about 25 mN/m) and that, at lower concentrations, the succinylated sample was more efficient to increase the surface pressure. The general profile of the isotherm was not, however, drastically changed by succinylation. The slope of the increasing part and the π_e plateau were not very much affected, neither was the lower concentration corresponding to the beginning of the plateau (16.7×10^{-6} g/mL).

The kinetics of surface adsorption were studied for three concentrations, one of them in the increasing linear part of the isotherm (4×10^{-6} g/mL) and the two others in the plateau region (20 and 40×10^{-6} g/mL). The equilibrium surface pressure (π_e) generally increased with the level of succinylation until a degree of modification of about 66% (Figure 5). A further increase of the level of succinylation (to 83%) decreased π_e . Succinylation resulted, moreover, in a decrease in the time (t_e) necessary for reaching the equilibrium. This effect was more pronounced at higher concentrations where t_e dropped from 28 h for the native protein to 18 h even after a weak modification, without further changes at higher degrees of succinylation. At the lowest concentration (4×10^{-6} g/mL), this effect was only observed above 66% modification, where t_e amounted to 21 h (Table I).

The analysis of the kinetics by the commonly used plots $\ln(d\pi/dt) = f(\pi)$ and $\ln(1 - \pi/\pi_e) = f(t)$ brought about four steps for the interface adsorption of each sample, as it was previously observed for model proteins (Graham,

Figure 3. Isoelectric focusing of the native and succinylated protein samples (elution buffer: Tris-SDS, pH 7): (a) native; (b) 29% (c) 65%, and (d) 93% succinylated samples.

1976) or oligomeric seeds globulins (Dagorn-Scaviner et al., 1986). According to the studies of Graham (1976)

the different steps were ruled successively by the diffusion of protein molecules to the interface (step 1), by their

Figure 4. Pressure isotherms (interface air/water): (O) native protein; (■) 83.5% succinylated protein.

Figure 5. Adsorption kinetics (interface air/water): (■) native protein; (□) 19.4%, (▲) 60.2%, (△) 66.0%, and (●) 83.5% succinylated proteins. Concentration 4×10^{-6} g/mL.

penetration into the film layer (step 2), and by molecular rearrangements within the adsorbed layer (step 3). A short former latent step (step zero) was also seen, which was previously described by other authors (Tornberg, 1978; Dagorn-Scaviner et al., 1986).

The characteristics of each step are summarized in Table I. The very short latent step did not exceed 3% of the total time of the kinetics for each sample. During this time, the native protein induced a change of surface pressure of 4.6%, whereas the modified proteins showed a higher effect increasing with the degree of modification from 7% at 19% succinylation to 15% at 83% succinylation.

The diffusion step was also relatively short (1.6–2.0%). The time for diffusion did not show a dependence on the level of modification (the 19% succinylated sample was an exception with 10%). The relative change of interfacial tension in this step was 25% for the native protein and significantly higher for the modified samples (31–60%). The rate constant of step 1 increased also with the degree of modification. Assuming that this step is mainly ruled by diffusion, it may be related to the fact that highly succinylated proteins contained components with lower molecular weight. The native globulin, as well as 90% of the 19% modified protein, sedimented around 12S and have an average rate constant around $1.5 \times 10^{-4} \text{ s}^{-1}$. On the other hand, when the degree of succinylation was over 60%, high amounts of 3S components appeared, leading to a higher rate constant of about $4.5 \times 10^{-4} \text{ s}^{-1}$ (Table I).

As generally observed, the energy barriers (ΔA) were

low for all samples in this step and did not show differences between the native and the modified proteins.

The penetration step (2) (Table I) was characterized by a long duration for the native and weakly (19%) succinylated protein, amounting to 50–60% of the total time, and a significantly shorter duration for the highly modified (higher than 60% succinylation) samples. The change of π_e by the native protein was the highest one during the total adsorption process, amounting to 66% of the change. The modified proteins caused a change of 30–40% of π_e without a direct relationship with the level of succinylation. The energy barriers (ΔA) and the rate constants (R) were increased by the highly (higher than 60%) modified proteins in this step.

The step of rearrangement (3) (Table I) was significantly shorter (33–36% of the total time) for the native or weakly modified proteins than for the highly (higher than 60%) succinylated ones (about 80% of the total time). The latter produced a pressure variation of 8–16% compared to the former ones that led to a change of 2–4% only. Table I shows also an increase of the energy barrier for all succinylated proteins irrespective of a clear dependence on the extent of modification. Nevertheless, the change of the rate constant (R) was less significant in this step.

Foaming Properties. Figure 6 and Table II show the kinetics of collapse of the foam of the native and succinylated rapeseed globulin in comparison with BSA. Taking into account the initial conductivity C_i as a measure of the foam capacity, one can notice that it was improved at high degrees of modification. C_i of highly modified proteins reached a value of $1100 \mu\text{S}/\text{cm}$ whereas it was only in the range $860\text{--}910 \mu\text{S}/\text{cm}$ for the native and weakly modified samples.

The two measures of foam stability ($t_{1/2}$ and FSI) represent different stages of foam destabilization. Whereas $t_{1/2}$ reflects an early stage of foam destabilization, FSI gives information about the state of the foam just before its collapse. $t_{1/2}$ describes therefore the capability of the protein to diffuse at the interface and to be adsorbed very quickly, stabilizing the air bubbles just after formation. Contrary to that, FSI, as a measure of coalescence phenomena, is more related to the protein film characteristics.

The modified proteins showed higher foam stability compared to the native one, especially for the higher degree of modification (Table II). The $t_{1/2}$, which was around 12.6 s for the native protein, reached, respectively, 36 and 41 s in the case of the 65% and 93% succinylated samples. It still remained lower than the corresponding value of BSA (75 s). The FSI of the native globulin, amounting to 49 min, increased just after a moderate level of succinylation of 28% and reached values of 64–68 min, which were higher than that of BSA (62 min). It seems consequently that succinylation mainly improves the efficiency of the protein in limiting the coalescence; the ability for stabilizing the air bubbles just after their formation is not so much increased in proportion, taking BSA as a reference.

These results were also discussed on the basis of the rate constant values (α), obtained by using the $\ln [(C - C')/C_i]$ versus time plot as previously explained (Figure 1b). Step 1 considered as mainly related to the drainage, because most of this phenomenon generally took place just after foam formation, is characterized by a rate constant related to the ability of the protein to adsorb quickly at the interface and to stabilize the air bubbles. On the other hand, step 3 reflects the collapse of the air bubbles

Table I. Characteristics of the Adsorption Kinetics of the Rapeseed Globulin (Native and Succinylated) at the Air/Water Interface (Buffer, Phosphate, pH 9.2; Concentration, 4×10^{-6} g/mL)

protein	step	$\Delta\pi$, mN/m	$\Delta\pi/\pi_e$, %	Δt , min	$\Delta t/t_e$, %	ΔA , $\text{\AA}^2$	R , $10^{-4}/\text{s}$	τ	π_e , mN/m	t_e , h
native	0	0.8	4.6	10.5	0.7					
	1	4.3	25.0	26.5	1.8	20	1.9	1.4 h	17.2	24
	2	11.4	66.3	883.0	61.3	127	0.6	4.6 h		
	3	0.7	4.1	520.0	36.1	251	0.4	6.9 h		
succinylated, 19.4%	0	2.0	10.5	39.0	2.7					
	1	11.3	59.5	146.0	10.1	28	1.2	2.3 h	19.0	24
	2	5.3	28.0	775.0	53.8	238	0.6	4.6 h		
	3	0.4	2.1	480.0	33.3	909	0.4	6.9 h		
succinylated, 60.2%	0	1.7	7.7	15.2	1.0					
	1	10.5	47.7	30.0	2.0	0	4.0	41.6 min	22.0	25
	2	7.0	31.8	200.0	13.3	158	1.0	2.7 h		
	3	2.8	12.7	1255.0	83.6	432	0.4	6.9 h		
succinylated, 66.0%	0	2.0	8.7	12.2	0.9					
	1	9.4	41.2	23.3	1.8	13	4.3	38.7 min	22.8	21
	2	9.5	41.6	260.0	20.6	146	1.1	2.5 h		
	3	1.9	8.3	965.0	76.6	620	0.7	3.9 h		
succinylated, 83.5%	0	3.2	15.3	12.4	1.0					
	1	7.8	37.3	19.4	1.6	13	5.0	33.0 min	20.9	20
	2	6.5	31.1	158.0	13.1	199	1.1	2.5 h		
	3	3.4	16.2	1010.0	84.1	350	0.5	5.5 h		

^a Key: $\Delta\pi$, contribution of the step to the increase of interfacial pressure; π_e , equilibrium interfacial pressure; t_e , equilibrium time; ΔA , energy barrier; R , rate constant; τ , relaxation time.

Figure 6. Kinetics of foam destabilization (buffer, pH 9.2): (●) bovine serum albumin; (■) native globulin; (□) 28%, (▲) 65%, and (△) 93% succinylated proteins.

and is more related to the behavior of the adsorbed film. As shown in Table II, it was clearly observed from α_1 , α_2 , and α_3 values that an increasing degree of succinylation makes easier the adsorption of the protein and stabilization of bubbles. These α values were, however, higher than the BSA ones, even for the highly succinylated samples; that agrees with our previous data relative to $t_{1/2}$. The results corresponding to step 3 showed that collapse is more effective for native and weakly modified proteins. The electrostatic repulsions between bubbles are more important for highly modified proteins because of a higher charge of the adsorbed film.

Emulsion Stabilization. Figure 7 shows the development of flocculation-creaming of the native and the differently modified proteins in the water/dodecane system. The analysis of each step of the kinetics is reported in Table III.

According to the speed of water-phase separation, and the amount of separated water, the proteins studied fell into two groups, one of which comprises the native and weakly (28%) modified proteins having a weak stabilizing effect. The second group includes the highly ($\geq 65\%$) modified samples showing a greater effect of stabilization. BSA as a standard protein gave intermediate results.

The analysis of the three steps of the kinetics revealed characteristic differences in the kinetic constants (β) and duration time (Δt). Generally, the duration time of steps 1 and 2 (Δt_1 and Δt_2) corresponding to the flocculation-creaming process was higher for the modified proteins, whereas the third step did not show differences in Δt_3 . The differences between β of the various proteins studied are most pronounced in the first step, reflecting a significantly quicker destabilization of the emulsion in the presence of the native protein ($\beta_1 = 14.1 \times 10^{-4} \text{ s}^{-1}$) than for the highly succinylated samples ($\beta_1 = 3.5 \times 10^{-4} \text{ s}^{-1}$), whereas the weakly modified protein gave an intermediate value ($\beta_1 = 8.3 \times 10^{-4} \text{ s}^{-1}$). β_1 and Δt_1 of BSA were similar to those of the highly succinylated proteins. The second step of kinetics showed only small differences of β_2 between the unmodified ($8.3 \times 10^{-4} \text{ s}^{-1}$) and the highly modified samples ($7.5 \times 10^{-4} \text{ s}^{-1}$), whereas the moderately succinylated samples had values of $(3.8\text{--}5.8) \times 10^{-4} \text{ s}^{-1}$. Similar behavior was observed in the third step; β_3 was $1.6 \times 10^{-4} \text{ s}^{-1}$ for the 29% modified protein whereas the highly modified samples led to values ($\beta_3 = (2.5\text{--}2.8) \times 10^{-4} \text{ s}^{-1}$) closer to that obtained for the native globulin ($\beta_3 = 3.3 \times 10^{-4} \text{ s}^{-1}$).

A comparison of the volume fraction Φ_e of the apolar phase in the creamed phase showed the lowest values for the highly modified proteins ($\Phi_e = 0.47\text{--}0.49$) and nearly comparable values between those of the native globulin (0.55) and BSA (0.53), which underscores the higher emulsion stabilization efficiency of the rapeseed globulin at a high level of succinylation.

DISCUSSION

The change in the functional properties of the 12S globulin depending on the degree of succinylation can be explained by the structural alterations of the proteins taking into account the change in molecular mass (disociation) and shape (unfolding) as well as the increase of negative charge resulting in an increase of hydration of the protein molecules. Generally, these alterations improve the surface activity at the air/water interface as well as the foaming properties and emulsion stability of the native protein under the conditions applied in this paper.

From a structural point of view, one can expect that

Table II. Foaming Properties (Buffer, Phosphate, pH 9.2; Concentration, 1 mg/mL)

protein	degree of succinylation	C_i , $\mu\text{S/cm}$	$t_{1/2}$, s	FSI, min	1st step		2nd step		3rd step	
					Δt_1 , min	α_1 , $10^{-4}/\text{s}$	Δt_2 , min	α_2 , $10^{-4}/\text{s}$	Δt_3 , min	α_3 , $10^{-4}/\text{s}$
BSA	0	1060	75.0	61.2	0.83	119.0	1.67	36.3	7.50	11.0
native globulin	0	870	12.6	49.0	0.25	238.0	0.95	89.0	7.80	30.8
succinylated globulins	28	860	16.5	68.0	0.40	225.0	0.80	107.6	2.80	41.0
	29	910	23.1	65.6	0.50	122.0	0.50	64.1	4.00	21.3
	65	1120	36.5	68.0	0.50	159.0	0.70	73.5	3.80	25.8
	83	970	19.5	94.6	0.60	152.0	0.60	83.0	4.80	28.6
	93	1160	41.0	64.0	0.25	141.0	0.80	63.8	3.80	18.5

^a Key: C_i , initial conductivity; $t_{1/2}$, time corresponding to $C_i/2$; FSI, foam stability index ($C_0\Delta t/\Delta c$); Δt_i , duration of each step; α_i , rate constant for each step.

Figure 7. Kinetics of emulsion destabilization (buffer, pH 9.2): (●) bovine serum albumin; (■) native globulin; (□) 28%, (▲) 65%, and (△) 93% succinylated proteins.

highest change of functionality of the protein above the 65% critical level of modification. Most of the rapeseed globulin is dissociated, leading to unfolded subunits. This unfolding results in a viscosity increase and in interactions between the polypeptide chains that may overcome the repulsive effects of the negative charge (Schwenke et al., 1980, 1986; Prah and Schwenke, 1986). These effects are to be discussed on the basis of the interface adsorption data, in interpreting the stabilization properties of the highly succinylated protein for foams and emulsions.

The differences in the adsorption kinetics of the native and the succinylated samples reflect indeed the structural changes and the charge increase (Table I). Increasing the degree of modification did not modify very much the duration of the step 0. On the other hand, it increases significantly the contribution of this step to decrease the surface tension. According to Blank et al. (1975), this preliminary step mainly results from an electrostatic barrier to adsorption, due to water molecules oriented at the interface. Adsorption of the first protein molecules disorganizes this water interface layer and induces positive surface pressure. Therefore, this last phenomenon seems to be more efficient for succinylated protein, probably because of their charge.

The comparison of the behavior of the native and weakly (19%) modified samples in the other steps of kinetics points to a significant influence of small changes in the charge and size of the protein molecules. Those induced a remarkable change of surface tension in the diffusion and penetration steps ($\Delta\pi/\pi_0$ amounts to 60 and 28%, respectively, of the total) and a significant increase of the energy barrier in the step of penetration (ΔA_2) and rearrangement (ΔA_3). The latter ones ought to be caused by electrostatic repulsion.

In the case of highly succinylated samples, the energy barriers (ΔA_2 , ΔA_3) were smaller in these steps than for the weakly modified ones, although the negative charges are much more increased. Apparently, the electrostatic barrier due to these charges was compensated by the decay of the big oligomeric 12S molecule into dissociated molecules that decreased the steric barrier. Furthermore, protein-protein interaction between hydrophobic regions exposed after the unfolding of the protein might be taken into account.

The more obvious phenomenon is the existence of small dissociation products that led to an increase of the speed constant in the first step of kinetics corresponding to the diffusion. Moreover, the high charge density of the strongly succinylated proteins is obviously responsible for prolongation of the third step consisting of the rearrangement of the molecules at the interface.

Since an investigation of succinylated oligomeric proteins by means of comparable methods is not described in the literature, the present results were compared to the only ones available on plant seeds oligomeric proteins reported by Dagorn-Scaviner et al. (1986) and concerning the interface adsorption kinetics of pea legumin (12S protein) and vicilin (7S protein). The π_e plateau value at the air/water interface was found around 25 mN m^{-1} for vicilin and legumin, which is very close to the values obtained for native or highly modified rapeseed 12S globulin. Moreover, it was concluded, as here for the dissociated polypeptide chains, that the higher diffusion rate of vicilin to the interface compared to legumin was due to its lower molecular weight.

On the basis of the isotherms (Figure 4), it seems that the equilibrium state of adsorption was not so different between the native and the 83% succinylated proteins. The interface areas per molecule in the adsorbed monolayer, calculated from the slope of the adsorption isotherm (Graham, 1976), were 0.81 and 1.25 nm^2 , respectively. These values are close to the corresponding one obtained by Dagorn-Scaviner et al. (1986) for pea legumin (1.04 nm^2).

From these results on adsorption, we may conclude that succinylation affects more the kinetic parameters (rate constant of diffusion, energy barriers) of the adsorption, due to the size and charge of the modified proteins, than the equilibrium characteristics. The foaming and emulsifying properties of the succinylated samples were discussed with regard to this conclusion.

It is interesting to note that the succinylation exerts various effects on the foaming capacity and the foam stability. The foaming capacity (C_i) (Table II) increased with a higher degree of succinylation; it is related to the higher rate constant for the diffusion of the dissociated components to the interfacial layers as established by the kinetic studies on interface adsorption (Table I). This

Table III. Emulsifying Properties (Buffer, Phosphate, pH 9.2; Concentration, 1 mg/mL)

protein	degree of succinylation	V_e , mL	Φ_e	1st step		2nd step		3rd step	
				Δt_1 , min	β_1 , $10^{-4}/s$	Δt_2 , min	β_2 , $10^{-4}/s$	Δt_3 , min	β_3 , $10^{-4}/s$
BSA	0	5.3	0.	15	3.6	13	6.8	32	2.8
native globulin	0	5.5	0.55	7	14.1	7	8.3	46	3.3
succinylated globulins	28	5.7	0.58	13	8.6	17	5.8	30	2.1
	29	5.8	0.59	11	9.3	15	3.8	34	1.6
	65	4.9	0.49	22	3.6	8	0.8	30	2.5
	66	5.4	0.54	17	5.3	7	1.5	36	3.8
	83	4.6	0.46	15	4.0	13	7.5	32	2.8
	93	4.7	0.47	20	3.5	6	7.5	34	2.5

* Key: V_e , aqueous phase volume at equilibrium; Φ_e , volume fraction of organic phase in the creamed phase ($\Phi_e = 2.5 \text{ mL}/(10 \text{ mL} - V_e)$); Δt_i , duration of each step; β_i , rate constant for each step.

kinetic parameter could explain the higher ability of these molecules in stabilizing the air bubbles just injected through the protein solution. The better foam-stabilizing effects of highly succinylated proteins (increasing $t_{1/2}$ and FSI; lower α values) (Table II) have to be related to the electrostatic repulsive effects between the air bubbles, which are due to the increasing charge of the adsorbed proteins. That is probably reinforced by the better rheological properties of the interfacial layers according to the increased viscosity of the succinylated proteins. Dissociation into subunits, significant increase of the negative charge, and the viscosity occurring for degrees of succinylation above 65% explain the fact that only highly modified proteins exhibit improved foaming properties.

The peculiar behavior of the 83% succinylated samples (Table II) needs an explanation considering a simultaneous influence of particle size and shape as well as charge effects. Its low stabilization ability in the first steps of foam breaking (decrease of $t_{1/2}$) could be related to its lower efficiency to decrease the surface tension (Figure 5). Its high stabilization effect in the last step (highest value of FSI) has to be discussed with regard to the increase of the negative charge. It might signify that, for highly succinylated protein, an optimized balance has to be found between size and charge for generating improved foaming properties. It is probably the case for the 93% modified protein sample.

Although it was found in some succinylated proteins a decrease of foam stability with increasing degree of modification (Schwenke and Rauschal, 1983; Schwenke et al., 1983b), this effect was not observed in the present study for the rapeseed globulin. This result agrees with the previous data reported by Nitecka and Schwenke (1986) on the same protein. That may be caused by structural peculiarities of the rapeseed globulin and a beneficial effect of the distribution of hydrophilic and hydrophobic groups in the sequence areas exposed by the unfolding of the protein.

The efficiency of the succinylated samples in stabilizing emulsions can also be explained by the increase of the negative charges, as previously done for foam stability. The lower values of the creaming-flocculation rate (β_1 , β_2) (Table III) reflect repulsion forces between dodecane droplets, stronger when the adsorbed protein layers are charged. The efficiency of succinylated proteins in retarding emulsion breaking has also to be related to the decreasing values of the volume fraction of the organic phase in the creamed phase (Φ_e) (Table III). Lower Φ_e values mean that the final packing of the dodecane droplets is less dense for modified proteins and indicate consequently that the effective thickness of the interfacial layer is larger due to hydration and charge. Higher hydration of the protein layer is enhanced by the unfolding of the polypeptide chains. This effect, more pronounced

at a high degree of succinylation, therefore contributes to the better stabilizing efficiency of the highly modified proteins. The abnormal behavior of the 66% succinylated sample might be caused by its distribution in dissociated products (Figure 2) (12S besides 7S and 3S in about equal proportions) and a possibly unequal charge distribution in the undissociated and dissociated forms. It was previously observed that emulsifying efficiency of mixed proteins could be lower compared to that of the proteins alone (Dagorn-Scaviner et al., 1987). This result was explained by the authors by "the molecular heterogeneity of the interfacial layer, which might induce higher fragility of the film".

In conclusion, the kinetic approach applied in this paper opens new possibilities of derivation of structure-function relationships for both the foaming and emulsifying properties. The succinylated proteins appeared to be more efficient emulsifying and foaming agents, mainly due to their charge and size. Whereas the effect of modification on the equilibrium interfacial pressure is fairly weak, the kinetics aspects of adsorption seem to be very important in explaining these functional properties, this fact being very consistent with our previous results on pea globulins (Dagorn-Scaviner et al., 1987). Moreover, the charge and the unfolding as well as the increased viscosity of the modified proteins induced positive effects on the stability of foams and emulsions.

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LITERATURE CITED

- Barber, K. J.; Wharthesen, J. J. Some functional properties of acylated wheat gluten. *J. Agric. Food Chem.* 1982, 30, 930-939.
- Beuchat, L. R. Functional and electrophoretic characteristics of succinylated peanut flour protein. *J. Agric. Food Chem.* 1977, 25, 258-261.
- Bhatty, R. S.; MacKenzie, S.; Finlayson, A. J. The proteins of rapeseed (*Brassica napus* L.) soluble in salt solutions. *Can. J. Biochem.* 1968, 46, 1191-1200.
- Blank, M.; Lee, B. B.; Britten, J. S. Adsorption kinetics of albumin monolayers. *J. Colloid Interface Sci.* 1975, 50, 215-221.
- Booth, V. H. Problems in the determination of FDNP-available lysine. *J. Sci. Food Agric.* 1971, 22, 658-666.
- Canella, M.; Castriotta, G.; Bernardi, A. Functional and physicochemical properties of succinylated and acylated sunflower protein. *Lebensm. Wiss. Technol.* 1979, 12, 95-101.
- Choi, Y. R.; Lusas, E. W.; Rhee, K. C. Succinylation of cotton flour: effect on the functional properties of protein isolates prepared from modified flour. *J. Food Sci.* 1981, 46, 954-955.

- Dagorn-Scaviner, C. Etude des propriétés tensioactives des globulines du pois. Application aux isolats protéiques utilisés comme agents d'émulsification ou de moussage. Thesis, Université de Nantes, France, 1986.
- Dagorn-Scaviner, C.; Gueguen, J.; Lefebvre, J. A comparison of interfacial behaviours of pea (*Pisum sativum* L.) legumin and vicilin at air/water interface. *Die Nahrung* 1986, 30, 337-347.
- Dagorn-Scaviner, C.; Gueguen, J.; Lefebvre, J. Emulsifying properties of pea globulins as related to their adsorption behaviours. *J. Food Sci.* 1987, 52, 335-341.
- Dalgalarondo, M.; Robin, J. M.; Azanza, J. L. Subunit composition of the globulin fraction of rapeseed (*Brassica napus* L.). *Plant Sci.* 1986, 43, 115-124.
- Franzen, K. L.; Kinsella, J. E. Functional properties of succinylated and acetylated soy proteins. *J. Agric. Food Chem.* 1976, 24, 788-795.
- Goding, C. A.; Bhatti, R. S.; Finlayson, A. J. The characterization of the 12S globulin from rapeseed and its glycoprotein component. *Can. J. Biochem.* 1970, 48, 1096-1105.
- Graham, D. E. Structure of adsorbed protein films and stability of foams and emulsions. Ph.D. Thesis CNAA, London, 1976.
- Graham, D. E.; Phillips, M. C. The conformation of proteins at interfaces and their role in stabilizing emulsions. In *Theory and practice of emulsion technology*; Smith, A. L., Ed.; Academic Press: London, 1976; pp 75-98.
- Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. I. Kinetics of adsorption and surface denaturation. *J. Colloid Interface Sci.* 1979a, 70, 403-414.
- Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. II. Adsorption isotherms. *J. Colloid Interface Sci.* 1979b, 70, 415-426.
- Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. III. Molecular structure of adsorbed film. *J. Colloid Interface Sci.* 1979c, 70, 427-439.
- Habeeb, A. Determination of the free amino groups in proteins using trinitrobenzenesulfonic acid. *Anal. Biochem.* 1966, 14, 328-336.
- Johnson, E. A.; Brekke, C. J. Functional properties of acylated pea protein isolates. *J. Food Sci.* 1983, 48, 722-725.
- Kato, A.; Takahashi, A.; Matsudomi, N.; Kobayashi, K. Determination of foaming properties of proteins by conductivity measurements. *J. Food Sci.* 1983, 48, 62-65.
- Kinsella, J. E. Functional properties, of proteins in foods: a survey. *CRC Crit. Rev. Food Sci. Nutr.* 1976, 7, 219-280.
- Martin, R. G.; Ames, B. N. A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. *J. Biol. Chem.* 1961, 236, 1372-1379.
- Mita, T.; Ishida, E.; Matsumoto, H. Physicochemical studies on wheat protein foams. II. Relationships between bubble size and stability of foams prepared with gluten and gluten components. *J. Colloid Interface Sci.* 1978, 59, 172-178.
- Nitecka, E.; Schwenke, K. D. Functional properties of plant proteins. Part 8: Effect of succinylation on some functional properties of the main globulin fraction from rapeseed (*Brassica napus* L.). *Die Nahrung* 1986, 30, 969-974.
- Phillips, M. C. The conformation and properties of proteins at liquid interfaces. *Chem. Ind.* 1977, 5, 170-177.
- Phillips, M. C. Protein conformation at liquid interfaces and its role in stabilizing foams and emulsions. *Food Technol.* 1981, 35, 50-57.
- Plietz, P.; Damaschun, H.; Müller, J. F.; Schwenke, K. D. The structure of 11S globulins from sunflower and rape seed. A small angle X-Ray scattering study. *Eur. J. Biochem.* 1983, 130, 315-320.
- Prahl, L.; Schwenke, K. D. Functional properties of plant proteins. Part 7. Rheological properties of succinylated protein isolates from faba beans (*Vicia faba* L.). *Die Nahrung* 1986, 30, 311-318.
- Raab, B.; Schwenke, K. D. Simplified isolation procedure for the 12S globulin and the albumin fraction from rapeseed (*Brassica napus* L.). *Die Nahrung* 1984, 28, 863-866.
- Rauschal, E. J.; Linow, K. J.; Pähz, W.; Schwenke, K. D. Chemische Modifizierung von Proteinen. 8. Mitt. Beeinflussung physikochemischer und funktioneller Eigenschaften von Proteinen aus Ackerbohnen durch Succinylierung. *Die Nahrung* 1981, 25, 241-248.
- Schwenke, K. D.; Rauschal, E. J. Functional properties of plant proteins. Part 6. Some functional properties of succinylated protein isolates from sunflower seed (*Helianthus annuus* L.). *Die Nahrung* 1983, 27, 1015-1024.
- Schwenke, K. D.; Raab, B.; Schultz, M. Über Samenproteine. 6. Mitt. Konformationsanalyse von Globulinen und Albuminen aus den Samen von Sonnenblumen (*Helianthus annuus* L.) und Raps (*Brassica napus* L.) durch Bestimmung des Circular dichroismus im fernen UV. *Stud. Biophys.* 1975, 51, 161-172.
- Schwenke, K. D.; Schultz, M.; Linow, K. J.; Gast, K.; Zirwer, D. Hydrodynamic and quasi-elastic light scattering studies on the 12S globulin from rapeseed. *Int. J. Peptide Protein Res.* 1980, 16, 12-18.
- Schwenke, K. D.; Raab, B.; Linow, K. J.; Pähz, W.; Uhlig, J. On seed proteins. Part 13: Isolation of the 12S globulin from rapeseed (*Brassica napus* L.) and characterisation as a "neutral" protein. *Die Nahrung* 1981, 25, 271-280.
- Schwenke, K. D.; Raab, B.; Plietz, P.; Damaschun, G. The structure of the 12S globulin from rapeseed (*Brassica napus* L.). *Die Nahrung* 1983a, 27, 165-175.
- Schwenke, K. D.; Rauschal, E. J.; Robowsky, K. D. Functional properties of plant proteins. Part 4. Foaming properties of modified proteins from faba beans. *Die Nahrung* 1983b, 27, 335-350.
- Schwenke, K. D.; Rauschal, E. J.; Zirwer, D.; Linow, K. J. Structural changes of the 11S globulin from Sunflower seed (*Helianthus annuus* L.) after succinylation. *Int. J. Peptide Protein Res.* 1985, 25, 347-354.
- Schwenke, K. D.; Linow, K. J.; Zirwer, D. Modification of the oligomeric structure of 11S globulins from sunflower (*Helianthus annuus* L.) and rapeseed (*Brassica napus* L.) seeds by succinylation. *Die Nahrung* 1986, 30, 263-270.
- Tornberg, E. The application of the drop volume technique to measurements of the protein adsorption at interfaces. *J. Colloid Interface Sci.* 1978a, 64, 391-401.
- Tornberg, E. The interfacial behaviour of three food proteins studied by the drop volume technique. *J. Sci. Food Agric.* 1978b, 29, 762-776.
- Tornberg, E.; Granfeld, Y.; Hakansson, C. A comparison of the interfacial behaviour of three food proteins adsorbed at air/water and oil/water interfaces. *J. Sci. Food Agric.* 1982, 33, 904-917.
- Ward, A. F. H.; Tordai, L. Time dependence of boundary tensions of solutions. I. The role of diffusion in time effects. *J. Chem. Phys.* 1946, 14, 453-461.
- Ward, A. F. H.; Tordai, L. Time dependence of boundary tensions of solutions. IV. Kinetics of adsorption at liquid-liquid interface. *Recl. Trav. Chim. Pays-Bas* 1952, 71, 572-584.
- Zirwer, D.; Gast, K.; Welfe, H.; Schlesier, B.; Schwenke, K. D. Secondary structure globulins from plant seeds: a re-evaluation from circular dichroism measurements. *Int. J. Biol. Macromol.* 1985, 7, 105-108.