Structural Changes of Legumin from Faba Beans (*Vicia faba* L.) by Succinylation

Klaus Dieter Schwenke,*,† Constanze Knopfe,† Larissa M. Mikheeva,‡ and Valerij Y. Grinberg‡

University Potsdam, WIP-Forschungsgruppe Pflanzenproteinchemie, Arthur-Scheunert-Allee 114-116, D-14558 Bergholz-Rehbrücke, Germany, and Russian Academy of Sciences, Institute of Biochemical Physics, Vavilow Street 28, Moscow, Russia

The effect of progressive succinylation upon the conformation of faba bean legumin has been studied using chemical analysis, viscometry, analytical ultracentrifugation, UV and fluorescence spectroscopy, and differential scanning calorimetry (DSC). The protein dissociates gradually into 3 S subunits forming a 7 S intermediate. DSC measurements revealed a continuous loosening of the spacial structure with increasing degree of succinylation. Viscometric and spectroscopic studies indicate the presence of a particular conformational state at 60−80% succinylation, whereas a largely expanded structure was shown to exist in exhaustively succinylated legumin due to a cumulative effect of N- and O-succinylation.

Keywords: *Legumin; faba bean protein; succinylation; conformational changes*

INTRODUCTION

Succinylation has been widely used to improve the functional properties of plant proteins (Franzen and Kinsella, 1976; Kabirulla and Wills, 1982; Paulson and Tung, 1988; Gueguen et al., 1990). To elucidate structural reasons of the changed functionality of succinylated protein isolates, investigations of physicochemical properties of their purified main protein components, e.g., 11 S globulins, after succinylation have been performed (Shetty and Rao, 1978; Rao and Rao, 1979; Schwenke et al., 1985, 1990; Kim and Kinsella, 1986). Though some data on physicochemical changes of succinylated faba bean protein isolate have been reported (Rauschal et al., 1981), the knowledge about the molecular changes of their main protein component, legumin, after progressive succinylation is very scanty.

The present paper deals with the investigation of structural changes in faba bean legumin after progressive succinylation. This includes both the extent of chemical modification of functional groups, conformational changes measured by hydrodynamic and spectroscopic methods, and stability studies using differential scanning microcalorimetry.

MATERIALS AND METHODS

Materials. 2,4,6-Trinitrobenzenesulfonic acid (TNBS), *N*-acetyl tyrosine amide, DL-tyrosine methyl ester-hydrochloride were purchased from Sigma, Deisenhofen; 1-anilino-8-naphthalenesulfonic acid (ANS) was from Serva, Heidelberg, and buffer substances were from Merck, Darmstadt.

Standard phosphate buffer (0.05 M phosphate, pH 8.0, according to Sörensen brought to an ionic strength of 0.5 with sodium chloride) was used as solvent for the protein in all measurements with the exception of analytical ultracentrifugation and DSC measurements.

Methods. *Protein Preparation.* Legumin from faba beans (*Vicia faba* L. Minor, var. Scirocco) 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 3−5% as determined with SDS−polyacrylamide gel electrophoresis and analytical ultracentrifugation. Protein concentration was determined by means of a microbiuret method (Itzhaki and Gill, 1964). Native legumin was used for calibration, the protein content of which was determined using the Kjeldahl nitrogen content and the experimentally determined nitrogen−protein conversion factor amounting to 5.55 (Krause et al., 1996). The latter was modified for the succinylated samples taking into account the mass increase corresponding to the attached succinyl residues. It increased with increasing degree of N- and O-succinylation to 5.90 at complete blocking of all groups (Schwenke et al., 1990).

Legumin Modification and Determination of N- and O-Blocking. Succinylation was performed by stepwise addition of dry succinic anhydride to a solution of legumin in standard phosphate buffer as described previously by Schwenke et al. (1990).

The degree of N-succinylation was estimated by determination of the content of free amino groups using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (Fields, 1972) in a modified version as previously described (Krause et al., 1996).

The degree of O-succinylation (esterification of hydroxy amino acids) was estimated after splitting the ester bond by hydroxylamine and the formation of a hydroxamic acid/Fe^{3+} complex according to the method of Habeeb and Atassi (1969) in a modified version as follows:

To 28 μmol of legumin dissolved in 2 mL of 0.2 M boric acid−sodium borate buffer, pH 9, was added 2 mL of 2

* Corresponding author's present address: Institut für Angewandte Proteinchemie e.V., Stahnsdorfer Damm 81, D-14532 Kleinmachnow, Germany (e-mail food.proteins@prochem.pm.shuttle.de).

† University Potsdam.

‡ Russian Academy of Sciences.

S0021-8561(97)00984-9 CCC: $15.00
Published on Web 05/01/1998

M hydroxylamine hydrochloride, pH 10, and the solution was incubated at 40 °C for 2 h in a water bath. After being rapidly cooled, the solution was brought to pH 1 with 0.8 mL of 6 N HCl followed by 0.8 mL of 5% $FeCl_3$ solution in 0.1 N HCl. Protein precipitated and the absorbance of the supernatant after centrifugation was read at 540 nm. The number of ester groups was obtained from a calibration curve using DL-tyrosine methyl ester-hydrochloride as a standard.

The extent of O-tyrosinesuccinylation was estimated spectrophotometrically by measuring the absorption at 278 nm during the incubation in standard phosphate buffer taking into account the spontaneous hydrolysis of O-succinyltyrosine bond at pH > 5 (Riordan and Vallee, 1964). *N*-Acetyltyrosine amide was taken for calibration. The error in the determination of the degree of succinylation was 3%.

Surface Hydrophobicity. Surface hydrophobicity was determined from fluorescence probe measurements using ANS according to the method of Kato and Nakai (1980), modified as previously described in Schwenke et al. (1993).

Viscometry. Viscosity measurements were performed at 20 °C in an automatic viscometer type Viskotimer (LAUDA, Königshofen, FRG) using a 1.5 mL microcapillary. The protein content of the starting solution was about 1%. The viscosity was measured at four or five concentrations. The reduced viscosity (η_{sp}/c) was plotted against the protein concentration to obtain the intrinsic viscosity. The error of determination was 3%.

Analytical Ultracentrifugation. Sedimentation velocity analysis was performed in an analytical ultracentrifuge type 3170/B, MOM, Budapest, at 20 °C and 50 000 rpm using 0.05 M phosphate buffer, pH 8.0, with 0.1 M NaCl, $I = 0.22$. The protein concentration was 1%.

UV and Fluorescence Spectroscopy. UV spectroscopic investigations of protein solutions (1.00 g/L in standard phosphate buffer) were carried out with a Lambda 2 dual-beam spectrophotometer from Perkin-Elmer, Überlingen. The spectra were measured with a scanning rate of 0.1 nm/s and slit of 2 nm. The difference-second-derivative spectra (second derivation of the difference succinylated legumin − native legumin) were calculated over a distance of 4.9 nm.

Fluorescence emission spectra were obtained after excitation of the protein solutions (0.10 g/L in standard phosphate buffer) at 280 and 295 nm with an Aminco Bowman series 2 spectrometer, Rochester in 0.2 nm steps, and a scan rate of 0.2 nm/s. The band-pass was 4 nm in excitation and 2 nm in emission; the detector high voltage was 900 V.

Differential Scanning Calorimetry. Thermograms (about 0.5% protein in 0.05 M phosphate buffer, pH 8.0, with 1 M NaCl) were recorded over the temperature range 2−95 °C at a heating rate of 2 °C/min and excess pressure of 2.5 atm, using a differential adiabatic scanning microcalorimeter DASM 4 (Biopribor, Russia). A temperature corresponding to the thermogram maximum was taken as the protein denaturation temperature. A baseline of the thermogram in the denaturation range and a denaturation enthalpy were calculated by spline interpolation and numerical integration methods, respectively.

RESULTS

Succinylation of Functional Groups. Extensive blocking of protein amino groups (95−98% N-succinylation) was obtained with a 40−50 molar excess of succinic anhydride, while 65−70% of the amino groups were succinylated with a 5−7-fold excess of reagent (Figure 1a). Up to a degree of 80% N-succinylation, only traces of succinylated hydroxyl groups could be determined. An effective O-succinylation occurred after 93−95% of the amino groups were acylated (Figure 1b).

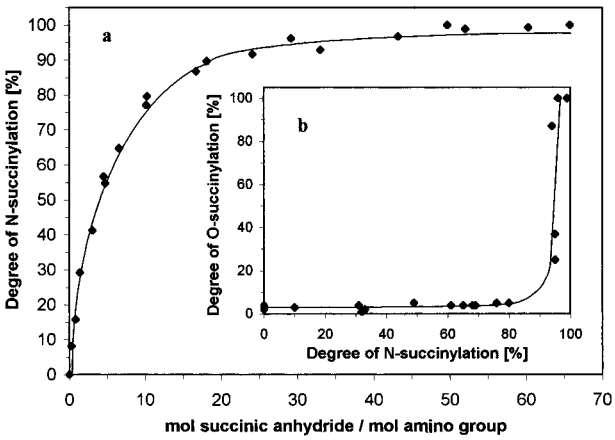

Figure 1. Succinylation of the amino groups in legumin as a function of the amount of succinic anhydride (a) and succinylation of hydroxyl groups in legumin at different steps of N-succinylation (b).

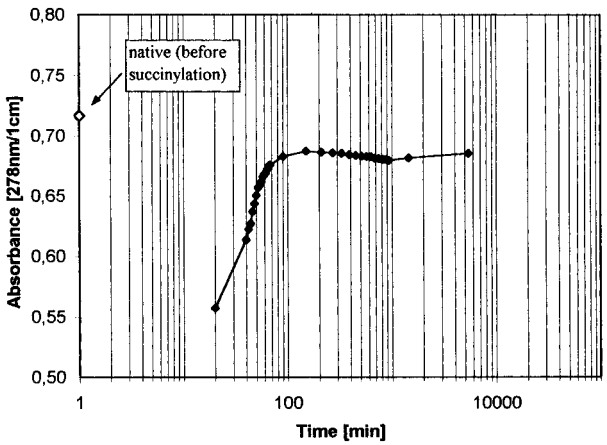

Figure 2. Changes of UV absorption (278 nm) of freshly prepared, exhaustively succinylated legumin due to the spontaneous desuccinylation of tyrosine residues during the incubation in standard phosphate buffer.

When the absorption of the protein at 278 nm was measured just after finishing an exhaustive succinylation (>95%) and compared with the data of unmodified legumin, a marked decrease was observed (Figure 2), which corresponded to the succinylation of 36 tyrosine residues/mol or 56% of the total tyrosine content (Schwenke et al., 1995). During 2 h incubation of the protein solution in standard buffer, the absorption approached the value of the native protein to 94% corresponding to 34 desuccinylated tyrosine residues.

Surface Hydrophobicity. A rectilinear increase of S_0 value corresponding to growing hydrophobicity with increasing degree of N-succinylation was observed up to 90−93% modification (Figure 3). At extremely high N-succinylation (>93%), where also a sudden increase of O-succinylation occurred, the hydrophobicity dropped down. This effect may be due to the dense distribution of negatively charged succinyl residues attached to amino and hydroxyl groups causing electrostatic repulsion, which inhibits the similarly charged ANS mol-

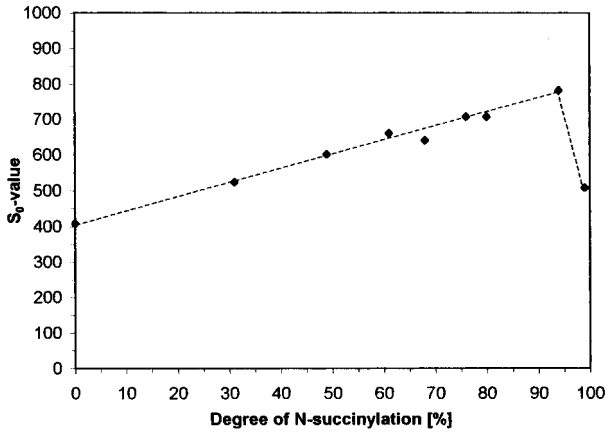

Figure 3. Surface hydrophobicity of legumin as a function of the degree of N-succinylation $c_{protein}$, 0.04 g/L; c_{ANS}, 0.05 g/L.

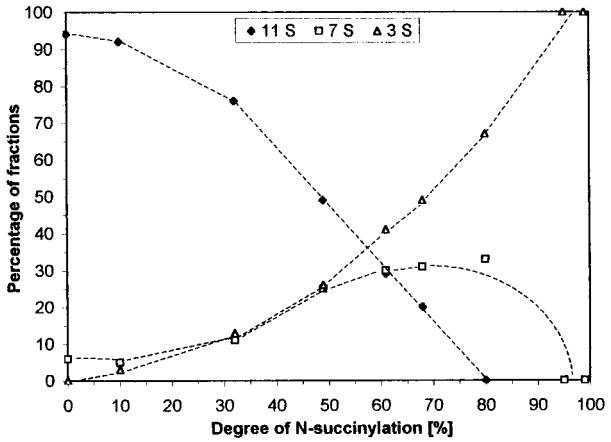

Figure 4. Percentage distribution of the different dissociated components of legumin as a function of the degree of N-succinylation from sedimentation velocity experiments.

ecules from approaching and undergoing interactions with exposed hydrophobic clusters of legumin.

Dissociation into Subunits. The dependence of the dissociation of legumin on the degree of N-succinylation is given in Figure 4. As other succinylated 11 S globulins (Schwenke et al., 1992), legumin also undergoes a dissociation into a 7 S component corresponding to the trimeric half-molecule and a 3 S component corresponding to the monomeric subunits. The latter appeared already at very low degrees of modification (about 10%). The 7 S component in the unmodified protein (about 5%) represents the vicilin fraction. The data given in Figure 4 indicate a simultaneaous dissociation of legumin into 7 S and 3 S components. A similar behavior has been reported also for the homologous protein from peanuts, arachin (Shetty and Rao, 1978), whereas a successive dissociation according to the scheme 11 S → 7 S → 3 S has been found for other 11 S globulins (Schwenke et al., 1992).

Intrinsic Viscosity. The intrinsic viscosity [η] of legumin increased from 6.1 cm^3/g at the unmodified state to 11.7 cm^3/g at 68% N-succinylation (Figure 5a). The discontinuous course of [η] between 60 and 80% succinylation coincides with the region where the 7 S intermediate has a maximum. A steep increase of [η] occurred at extremely high N-succinylation (93−98%), reaching a value of 19.3 cm^3/g. The reduced viscosity did not show a marked concentration dependence up to a modification of 75−80%, which became evident,

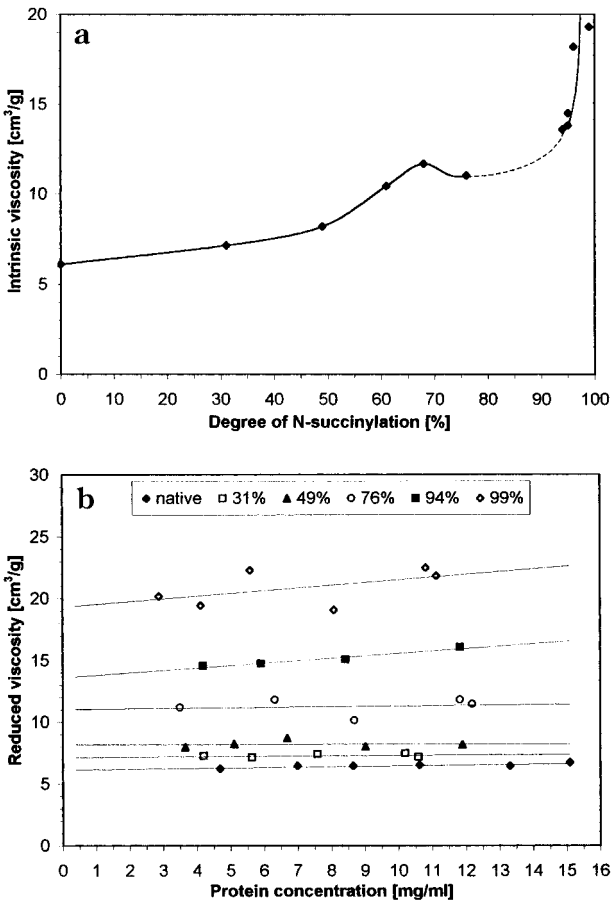

Figure 5. Viscosity of native and succinylated legumin in standard phosphate buffer: (a) intrinisic viscosity [η] as a function of the extent of succinylation and (b) concentration dependence of the reduced viscosity at different steps of succinylation.

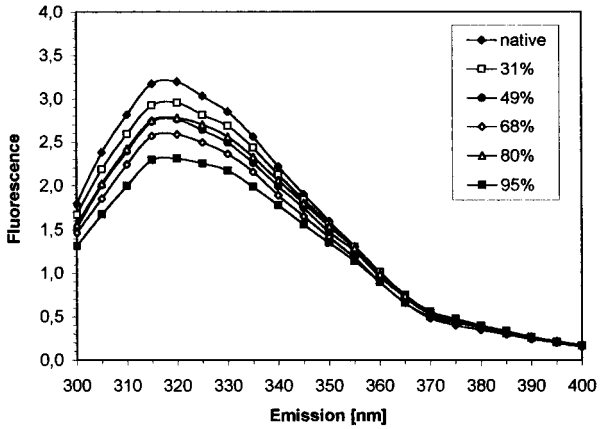

Figure 6. Fluorescence emission spectra of legumin at various steps of succinylation with an excitation at 280 nm.

however, at extremely high succinylation (> 90%) (Figure 5b).

UV and Fluorescence Spectroscopy. Figure 6 shows the fluorescence emission spectra of modified legumin at different steps of succinylation after excitation at 280 nm (Tyr + Trp excitation). The maximum position at 316 nm for the native legumin points to the dominating effect of tyrosine chromophores (Chen et al., 1969). A small red shift of the maximum was observed after succinylation amounting to 2−3 nm up to 50% succinylation, about 1 nm at 68−80% succinylation, and again 3 nm after exhaustive modification. The fluores-

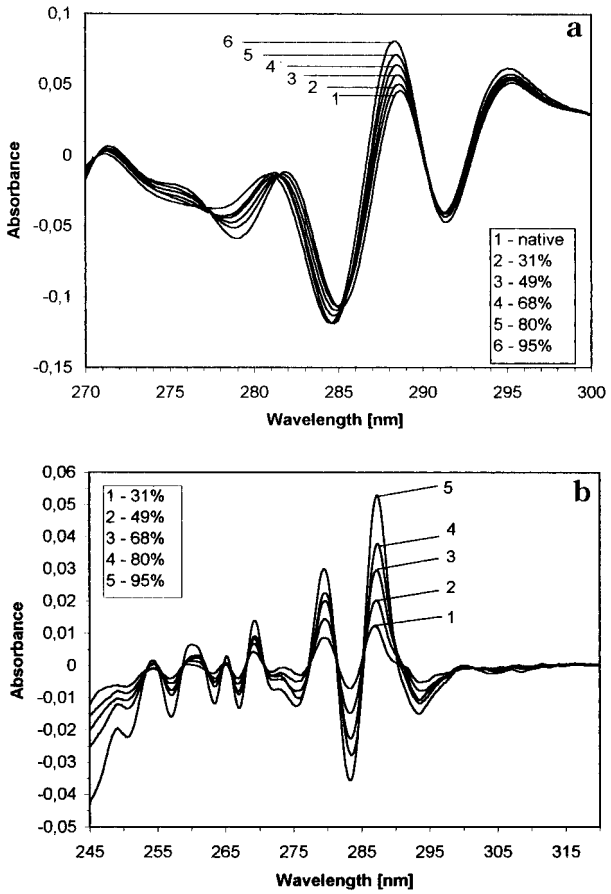

Figure 7. UV second-derivative spectra (a) and difference-second-derivative spectra (b) of variously succinylated legumin.

cence intensity decreased after succinylation up to a degree of 68%; it increased again at about 80% succinylation and dropped down markedly at 95% succinylation.

This behavior reflects the change of exposure of aromatic chromophores after succinylation but points also to a special conformational state of 70−80% succinylated legumin. Comparable changes have been found in the fluorescence emission spectra after excitation at 295 nm (principally Trp excitation). The changes in the fluorescence emission spectra differ from those of succinylated pea legumin at comparable degrees of modification. The latter showed highest intensity at about 60% succinylation and a drastic quenching at higher degrees (≤75%) of modification. Although both proteins reveal a high degree of homology (Plietz et al., 1987), they show differences in the primary structure and in the exposure of aromatic chromophores, which should result in a different response after succinylation.

The UV second-derivative spectra show a continuous blue shift of the bands in the tyrosine region (275−290 nm) with increasing extent of modification (Figure 7a) reflecting an increase in the unfolding processes of legumin. The difference-second-derivative spectra (Figure 7b) show marked changes both in the tryptophan region (>290 nm), tyrosine (275−290 nm), and phenylalanine (<275 nm) bands, which underline the involvement of all aromatic chromophores in the conformational changes after succinylation.

The ratio between two peak to peak distances in the UV second-derivative spectra, i.e., the peak to peak distance between the maximum at 287 nm and the minimum at 285 nm and the peak to peak distance

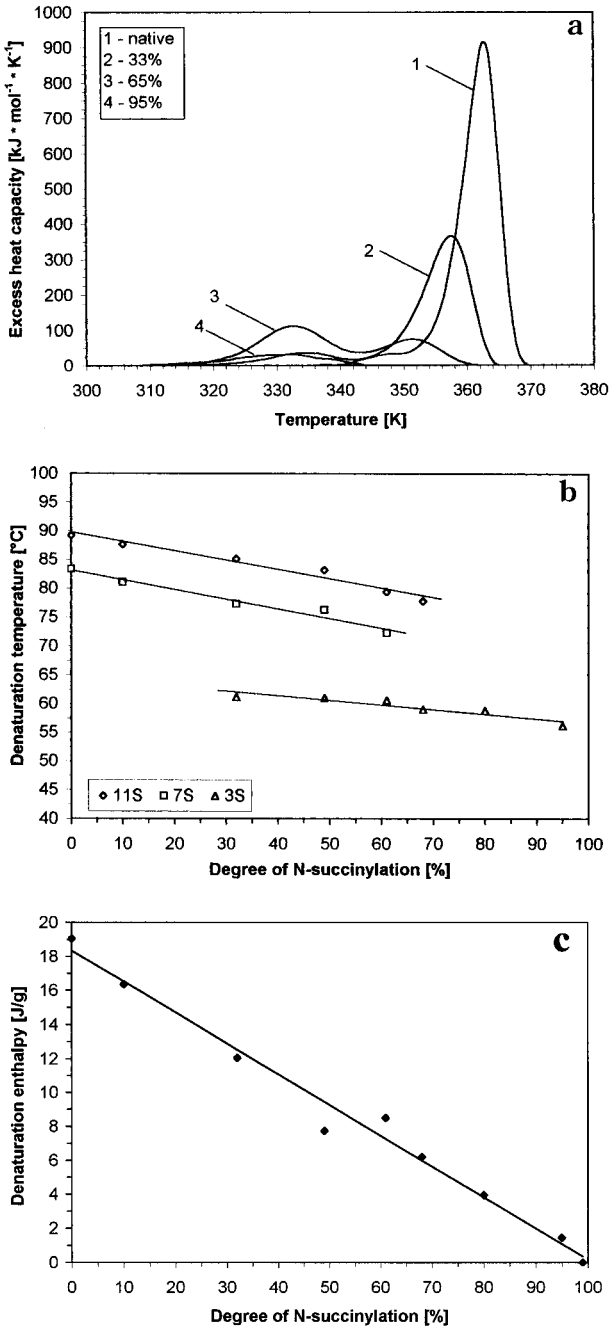

Figure 8. Differential scanning microcalorimetry (a) thermograms of legumin and succinylated legumin in 0.05 M phosphate buffer, pH 8 with 0.1 M NaCl, $I = 0.22$; the excess heat capacity is expressed per legumin hexamer; (b)denaturation temperature of the different components of legumin dissociation; (c)total specific enthalpy of denaturation as a function of the extent of modification.

between the maximum at 295 nm and the minimum at 290 nm, can be taken according to Ragone et al. (1984) as a measure of the ratio of tyrosine:tryptophan exposure. This ratio amounted to 1.51 for the native legumin. It increased to 1.97 with progressive succinylation up to 70% modification, decreased at about 80% modification to 1.85, and increased again after exhaustive succinylation to 2.02. Thus, both the fluorescence and UV spectroscopic data point to particular conformational changes of legumin after being succinylated to 70−80%.

Differential Scanning Microcalorimetry. Figure 8a gives the thermograms of native and varioulsy

succinylated legumin obtained in solutions of phosphate buffer with $I = 0.22$. The appearance of the different peaks corresponding to the products of dissociation coincides well with the results of ultracentrifugal analysis (Figure 4). The denaturation temperature $T_{d,max}$ of each component decreased linearly with increasing degree of modification, where $T_{d,max}$ (11 S) > $T_{d,max}$ (7 S) > $T_{d,max}$ (3 S) (Figure 8b). $T_{d,max}$ of native legumin was determined to 89.8 °C, whereas the lowest value of $T_{d,max}$ obtained for the dissociated 3 S component in exhaustively succinylated legumin was 53.6 °C. A continuous decrease of the total specific enthalpy of denaturation with increasing succinylation was also observed (Figure 8c).

DISCUSSION

The succinylation of amino groups in faba bean legumin can be realized practically completely (90−99%) with a moderate excess of succinic anhydride. A high reactivity of the amino groups against succinic anhydride has been also observed for the homologous legumin from pea (Schwenke et al., 1990). In contrast to that, the 11 S globulins from sunflower and rapeseed required an extremely high excess of reagent to become succinylated to more than 90% (Schwenke et al., 1986). These differences have been discussed on the basis of the availability of lysine residues in the constituent polypeptide chains (Schwenke et al., 1992) assuming that their exposed parts are the C-terminal regions of α-chains and the buried ones are the hydrophobic β-chains (Plietz et al., 1987). In faba bean legumin, the presence of two subunit types (A and B) should be taken into consideration. Both are homologous in the primary structure, but reveal a sufficiently great sequence difference and characteristic differences in the distribution of lysine residues over the polypeptide chains and the distribution of ordered structural regions (Bäumlein et al., 1986; Schlesier et al., 1990; Dudek et al., 1996).

However, a considerable percentage of lysine residues are located in both subunit types in the α-chains (α-A, 52%, α-B, 61%), the C-terminal regions of which contains 44% of the total lysines. Twenty-nine percent of the lysine residues in the α-A-chains and 23% of those in the α-B-chains are located in the flexible, unstructured C-terminus and ought to be exposed. Therefore, about 26% of the ϵ-amino groups should be preferentially succinylated, which coincides with the result of N-succinylation at low excess of reagent (Figure 1). The β-A- and β-B-chains show a distribution of lysine residues over the total amino acid sequence. The complete acylation of these residues ought to be possible only after the unfolding of the globular structure. In fact, the increase of the intrinsic viscosity at a degree of succinylation higher than 50−60% (Figure 5) signals a beginning molecular expansion.

In contrast to the large number of papers that reported on plant protein succinylation on the basis of N-acylation, there is very little information published that shows the participation of functional groups other than amino groups in the succinylation of plant storage proteins. In a previous paper on pea legumin, it was shown that, beside the N-succinylation, hydroxy amino acids were also succinylated (Schwenke et al., 1990). However, this O-succinylation (esterification) occurred only after the majority of amino groups were already acylated. This is consistent with findings on the O-succinylation of other globular proteins such as bovine serum albumin (Chang and Sun, 1978) and pepsinogen (Gounaris and Perlman, 1967). In the case of faba bean legumin, O-succinylation takes effect after more than 90% of the amino groups have been blocked (Figure 1b). Since the serine and threonine residues are distributed over the total primary structure of the α- and β-chains (Bäumlein et al., 1986; Schlesier et al., 1990), their total succinylation should result in an unusually high accumulation of negative charge, which leads to a complete dissociation and a large expansion of the dissociated subunits. This is reflected by the decrease in the total specific enthalpy of denaturation (Figure 8c) and the change in the intrinsic viscosity (Figure 5a,b). The increase in the intrinsic viscosity and the concentration dependence of the reduced viscosity after a succinylation of more then 90% of the amino groups (Figure 5a,b), i.e., in the region of excessive O-succinylation, give evidence for electrostatic effects that were not shielded completely at the ionic strength of 0.5 used in this study.

Since the succinylation of tyrosine residues does not lead to stable O-succinyl bonds, its influence on the total charge of the protein can be neglected. It is however interesting that 56% of the tyrosine residues of legumin were reversibly succinylated. This may point to a special exposure of these residues. Moreover, the limited tyrosyl succinylation of legumin might be due to steric hindrance in the largely unfolded but already densely succinylated polypeptide chains. The high negative charge density in extensely N-,O-succinylated legumin might also inhibit the approach of the negatively charged ANS molecules and in this way decrease the hydrophobicity coefficient S_0. This seems to have a marginal effect when only amino groups are succinylated. In this case, the hydrophobicity increased continuously up to 90% N-succinylation, which could be related to the progressive dissociation of legumin with the formation of 7 S and 3 S components each having a higher hydrophobicity.

The simultaneous formation of monomeric 3 S and trimeric 7 S components during the succinylation-induced dissociation of legumin raises the question about the different behavior of the constituent A and B subunits. It may be an arrangement of one of these subunits in the native legumin as 7 S trimers that remain existing during a rather large range of modification steps. A different arrangement of both subunit types should result in a quaternary structure with both isologous and heterologous binding domains between the subunit. A corresponding structural model has already been discussed (Plietz et al., 1983).

Ultracentrifugal, viscometric, and spectroscopic studies point to a special conformational state of modified legumin after being succinylated to 60−80%. To understand this fact, the possible charge influence on the conformation has been estimated on the basis of the amino acid composition of the legumin (Schwenke et al., 1995), taking into account an average degree of amidation of the acidic amino acids (Glu, Asp) of 50% (Bäumlein et al., 1986; Schlesier et al., 1990). Thus, an excess of 216 negative charges results per mol of legumin for a 80% N-succinylated protein. One might assume that the conformation of a 60−80% succinylated legumin corresponds to a structural state between that of the compact native protein and the largely expanded extensively succinylated protein that may be described as a molten globule-like structure (Ptitsyn, 1992). The molten globule state has been described as a special

conformation of acid-denatured proteins. Studying the physical properties of bovine serum albumin modified with succinic, maleic, or citraconic anhydrides, Jonas and Weber (1970) found an expanded state for a 65% modified sample, which is very similar in its physical properties to the protein expanded by electrostatic repulsion at pH 2.

Recently, the interfacial behavior and emulsifying properties of faba bean legumin and its variously (34%, 65%, 95%) succinylated derivatives have been studied (Krause et al., 1997). The results of surface tension measurements and surface shear rheometry and the properties of *n*-decane−water emulsions indicate an increased interfacial activity by succinylation whereby the 65% succinylated legumin was the most active derivative. Thus, the critical association concentration, i.e., the subphase concentration at which the equilibrium surface pressure was reached, and the occupied area calculated from the Gibbs isotherms gave the lowest values for the 65% succinylated sample. Spread and adsorbed films of legumin exhibited purely viscous behavior under shear stress whereby the viscosity strongly increased after succinylation and reached maximum values at 65% modification. This behavior was related to the increase of the overall amount of surface-active (sub)units and thus to the increase of the package density at the interface. These factors seem mainly to be responsible for the reduced surface tension and increased film viscosity. The purely viscous behavior of the interface layers supports the view that the legumin and its succinylated derivatives maintain a principle globular structure at the interface, i.e., no remarkable surface denaturation seems to occur that could lead to film elasticity by an entanglemant of flexible chains (Seifert and Schwenke, 1995; Krause et al., 1997). It is generally assumed that the surface denaturation of globular proteins after interfacial adsorption is limited by the orientation of the molecules at the interface (Narsimhan and Uraizee, 1992). Thus, the decrease in interfacial free energy is determined by the packing density of the molecules at the interface. Undoubtedly, the cohesive forces acting between the molecules adsorbed at the interface are distinctly strengthened by succinylation. Hydrophobic regions of dissociated molecules could be exposed by molecular rearrangement after adsorption and reinforcement of molecular interactions. Enhanced electrostatic repulsion and unfolding of the excessively succinylated legumin (95%) lead to a deterioration in surface functionality. It can be assumed that the high charge density maintains the molecule in a highly unfolded state and inhibits rearrangement processes at the surface by the repulsive effect (Subirade et al., 1992). In fact, a molten globule-like state has been shown to be the most surface-active conformational state of proteins (Dickinson and Matsumura, 1994).

The present DSC studies reveal a continuous decrease of the stability of legumin and its subunits with increasing level of succinylation. The decreasing specific enthalpy of denaturation reflects a continuous loosening of the compact globular structure and does not give a hint to a special behavior of "intermediary" (60−80%) modified legumin. To obtain a better insight into the corresponding structural state, further studies will be carried out.

In conclusion, step-by-step succinylation of legumin makes it possible to produce derivatives differing largely in the nature and extent of functional groups modification and consequently in their hydrophobicity and conformational state. The understanding of these structural changes is helpful for interpreting the changed surface functional properties of succinylated plant proteins.

ACKNOWLEDGMENT

We are highly indebted to Mrs. Angelika Krajewski, Mrs. N. V. Grinberg, Mrs. I. A. Golubeva, Mr. A. Y. Mashkevich, and Mr. A. L. Leontév for skilful technical assistance.

LITERATURE CITED

Bäumlein, H.; Wobus, U.; Pustell, J.; Kafatos, F. C. The legumin gene family: structure of a B type gene of *Vicia faba* and a possible legumin gene specific regulatory element. *Nucleic Acids Res.* **1986**, *14*, 2707−2720.

Chang, T.-S.; Sun, S. F. Structural studies on the succinylated bovine serum albumin. *Int. J. Peptide Protein Res.* **1978**, *11*, 65−72.

Chen, R. F.; Edelhoch, H.; Steiner, R. F. Fluorescence of proteins. In *Physical principles and techniques of protein chemistry*; Leach, S. J., Ed.; Academic Press: New York, 1969; pp 171−244.

Dickinson, E.; Matsumura, Y. Proteins at liquid interfaces: role of the molten globule state. *Colloids Surf. B: Biointerfaces* **1994**, *3*, 1−17.

Dudek, S.; Horstmann, C.; Schwenke, K. D. Limited tryptic hydrolysis of legumin from faba bean (*Vicia faba* L.): formation of an unequal subunit pattern. *Nahrung* **1996**, 40, 171−176.

Fields, R. The rapid determination of amino groups with TNBS. *Methods Enzymol.* **1972**, *25B*, 464−468.

Franzen, K. L.; Kinsella, J. E.Functional properties of succinylated and acetylated soy protein. *J. Agric. Food Chem.* **1976**, *24*, 788−795.

Gounaris, A. D.; Perlman, G. E. Succinylation of pepsinogen. *J. Biol. Chem.* **1967**, *242*, 2739−2745.

Gueguen, J.; Bollecker, S.; Schwenke, K. D.; Raab, B. Effect of succinylation on some physicochemical and functional properties of the 12 S storage protein from rapeseed (*Brassica napus* L.). *J. Agric. Food Chem.* **1990**, *38*, 61−69.

Habeeb, A. F. S. A.; Atassi, M. Z. Enzymic and immunochemical properties of lysozyme II. Conformation, immunochemistry and enzymic activity of a derivative modified at tryptophan. *Immunochemistry* **1969**, *6*, 555−566.

Itzhaki, R. F.; Gill, D. M. A micro-biuret method for estimating proteins. *Anal. Biochem.* **1964**, *9*, 401−410.

Jonas, A.; Weber, G. Partial modification of bovine serum albumin with dicarboxylic anhydrides. Physical properties of the modified species. *Biochemistry* **1970**, *9*, 4729−4735.

Kabirullah, M.; Wills, R. B. H. Functional properties of acetylated and succinylated sunflower protein isolate. *J. Food Technol.* **1982**, *17*, 235−249.

Kato, A.; Nakai, S. Hydrophobicity determinated by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.

Kim, S. H.; Kinsella, I. E. Effect of progressive succinylation on some molecular properties of soy glycinin. *Cereal Chem.* **1986**, *63*, 342−345.

Krause, J.-P.; Mothes, R.; Schwenke, K. D. Some physicochemical and interfacial properties of native and acetylated legumin from faba beans (*Vicia faba* L.). *J. Agric. Food Chem.* **1996**, *44*, 429−437.

Krause, J.-P.; Krägel, J.; Schwenke, K. D. Properties of intercial films formed by succinylated legumin from faba beans (*Vicia faba* L.). *Colloids Surf. B: Biointerfaces* **1997**, *8*, 279−286.

Narsimhan, G.; Uraizee, F. Kinetics of adsorption of globular proteins at an air−water interface.*Biotechnol. Progr*. **1992**, *8*, 187−196.

Paulson, A. T.; Tung, M. A. Rheologiy and microstructure of succinylated canola protein isolate. *J. Food Sci*. **1988**, *53*, 821−825.

Plietz, P.; Damaschun, G.; Müller, J. J.; Schwenke, K. D. The structure of 11 S globulins from sunflower and rape seed a small-angle X-ray scattering study. *Eur. J. Biochem*. **1983**, *130*, 315−320.

Plietz, P.; Drescher, B.; Damaschun, G.Relationship between the amino acid sequence and the domain structure of the subunits of the 11 S seed globulins. *Int. J. Biol. Macromol*. **1987**, *9*, 161−165.

Popello, I. A.; Suchkov, V. V.; Grinberg, V. Y.; Tolstoguzov, V. B. Isolation and purification of 11 S globulins from seeds of broad beans and peas. *Prikl. Biochem. Mikrobiol*. **1988**, *24*, 50−55 (in Russian).

Ptitsyn, O. B. The molten globule state. In *Protein Folding*; Creighton, T. E., Ed.; Freeman: New York, 1992; pp 243−300.

Ragone, R.; Colonna, G.; Balestrieri, C.; Servillo, L.; Irace, G. Determination of tyrosine exposure in proteins by second-derivative spectroscopy. *Biochemistry* **1984**, *23*, 1871−1875.

Rao, A. G. A.; Rao, M. S. N. Effect of succinylation on the oligomeric structure of glycinin. *Int. J. Pept. Protein Res*. **1979**, *14*, 307−312.

Rauschal, E. J.; Linow, K.-J.; Pähtz, W.; Schwenke, K. D. Chemische Modifizierung von Proteinen. 8. Mitt. Beeinflussung physikochemischer und funktioneller Eigenschaften von Proteinen aus Ackerbohnen durch Succinylierung (Chemical modification of proteins. Part 8. Effect of succinylation on physicochemical and functional properties of proteins from faba beans). *Nahrung* **1981**, *25*, 241−248.

Riordan, J. F.; Vallee, B. L. Succinylcarboxypeptidase. *Biochemistry* **1964**, *3*, 1768−1774.

Schlesier, B.; Bassüner, R.; Van Hai, N.; Müntz, K. The cDNA derived primary structure of two distinct legumin A subunit precursors from field bean (*Vicia faba* L.). *Nucleic Acids Res*. **1990**, *18*, 7146.

Schwenke, K. D.; Rauschal, E.; Zirwer, D.; Linow, K.-J. Structural changes of the 11 S globulin from sunflower seed (*Helianthus annuus* L.) after succinylation. *Int. J. Pept. Protein Res*. **1985**, *25*, 347−354.

Schwenke, K. D.; Linow, K.-J.; Zirwer, D. Modification of the oligomeric structure of 11 S globulin from sunflower (*Helianthus annuus* L.) and rape (*Brassica napus* L.) seeds by succinylation. *Nahrung* **1986**, *30*, 263−270.

Schwenke, K. D.; Zirwer, D.; Gast, K.; Görnitz, E.; Linow, K.-J.; Gueguen, J. Changes of the oligomeric structure of legumin from pea (*Pisum sativum* L.) after succinylation. *Eur. J. Biochem*. **1990**, *194*, 621−627.

Schwenke, K. D.; Zirwer, D.; Gast, K.; Linow, K.-J.; Görnitz, E.; Gueguen, J.; Subirade, M. Dissociation of 11 S globulins from plant seeds after succinylation—a problem of subunit interaction. In *Protein Interactions*; Visser, H., Ed.; VCH: Weinheim 1992; pp 233−252.

Schwenke, K. D.; Dudek, S.; Mothes, R.; Raab, B.; Seifert, A. Physicochemical and enzymatic studies on acetylated protein isolates from faba beans (*Vicia faba* L.). *Nahrung* **1993**, *37*, 258−268.

Schwenke, K. D.; Dudek, S.; Seifert, A.; Mothes, R.; Staatz, A. Isolation of faba bean legumin—a comparative study of various methods. *Nahrung* **1994**, *38*, 557−567.

Schwenke, K. D.; Staatz, A.; Dudek, S.; Krause, J.-P.; Noack, J. Legumin-T from faba bean legumin: isolation, partial characterization and surface functional properties. *Nahrung* **1995**, *39*, 193−202.

Seifert, A.; Schwenke, K. D. Improved approach for characterizing the coalescence stability of legumin stabilzed O/W emulsions by analytical ultracentrifugation. *Prog. Colloid Polym. Sci*. **1995**, *31*, 38.

Shetty, K. Y.; Rao, M. S. N. Effect of succinylation on the oligomeric structure of arachin. *Int. J. Pept. Protein Res*. **1978**, *11*, 305−313.

Subirade, M.; Gueguen, J.; Schwenke, K. D. Effect of dissociation and conformational changes on the surface behavior of pea legumin. *J. Colloid Interface Sci*. **1992**, *152*, 442−454.

Received for review November 18, 1997. Accepted March 10, 1998. The financial support of this work by a grant (Schw 462/4-1 and 436 Rus 113/47) of the Deutsche Forschungsgemeinschaft, Bonn, is gratefully acknowledged.

JF970984K