Action of Transglutaminase on an 11S Seed Protein (Pea Legumin): Influence of the Substrate Conformation

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The reactivity of globular seed protein (pea legumin) as substrate for transglutaminase is studied as a function of its conformation. Citraconylation of legumin induced, depending on the level of modification, a total or partial dissociation of the protein into subunits. The acylated legumins were used as substrates for guinea pig liver transglutaminase, and the transfer and hydrolytic activities were investigated. The ability of the amide groups of the glutaminyl residues to form ϵ-(γ-glutamyl)lysyl cross-links was studied, and their reactivity was compared with and without external amine, here [^{14}C]putrescine. Legumin characterized by a close-packed globular structure was shown to be a poor substrate for the enzyme despite its high content in glutaminyl and lysyl residues. On the other hand, citraconylation by inducing conformational changes drastically increased its reactivity toward transglutaminase. High levels of deamidation and quantity of acyl acceptors incorporated were reached; a polymerization, although low, still occurred.

INTRODUCTION

The modification of proteins by chemical agents has been extensively studied and was shown to be a very powerful tool for improving the nutritional as well as the functional properties of these macromolecules. Feeney and Whitaker (1985) suggested in their review that proteins could be tailored for better utilization in medical and cosmetic applications and also in food. In previous studies, we have shown the special interest of succinylation and glycosylation (Gueguen et al., 1990; Caer et al., 1990) for modifying the physicochemical properties and improving the functional characteristics of 11S-type seed proteins. However, for food uses, most of the chemical modifications could not be applied because of toxicity of the reagents, and the enzymatic way should be preferred. From this point of view, transferases can be potentially considered as interesting tools to modify proteins.

Transglutaminase (TGase, EC 2.3.2.13) has been extensively studied (Folk, 1980) and is known to catalyze the transfer of the γ-carboxamide group of glutaminyl residues in protein to primary amino groups in a variety of compounds. In vivo, the only known acyl acceptor is the ϵ-amino group of a lysyl residue (Folk and Finlayson, 1977). In the case of TGase (factor XIIIa), fibrin and α_2-antiplasmin were shown to be natural substrates. The formation of ϵ-(γ-glutamyl)lysyl cross-links enhances the mechanical strength of the blood clot and increased its lifetime in the plasma (Sakata and Aoki, 1980). The mechanism of this reaction has been extensively described from activation of plasma factor XIII to catalysis of the cross-links (Naski et al., 1991). In the case of intracellular transglutaminases, their physiological role has not been yet elucidated. However, from in vitro experiments many proteins were shown to be potential substrates for TGase, leading to the hypothesis concerning the TGase physiological role or to partial information concerning the specific requirements for a protein to be substrate.

Most proteins are bifunctional substrates with both glutaminyl donors and lysyl acceptors and are therefore able to cross-link. Although the ϵ-amino group of the lysine side chain is the only known in vivo acyl acceptor, other primary amines (putrescine, cadaverine) were shown to behave as acyl acceptors in vitro. Moreover, when no amine is available in the reaction mixture, water acts as acyl acceptor, leading to the hydrolytic cleavage of the amide group of glutaminyl residues (Neidle et al., 1958; Mycek et al., 1959). In 1960, Mycek and Waelsch (1960) showed that the hydrolysis or the replacement of the amide group occurred at the same place. Later on, Gorman and Folk (1980, 1984) pointed out that important determinants for enzyme reside in the linear sequence around the glutaminyl residue. However, as far as macromolecular substrates are concerned, only hypotheses on the conformational preferences of the enzyme have been made (Coussons et al., 1991). The glutamine should be located in a flexible region (Berbers et al., 1983), in a surface loop (Takashi, 1988), or in regions which are predicted to be reverse turns (Wold, 1985). The propensity of some food proteins to be modified by TGase has been studied (Motoki et al., 1986): milk proteins (Motoki et al., 1987), wheat gluten (Kurth and Rogers, 1984), and soybean proteins (Ikura et al., 1980) were shown to be substrates.

Despite their relatively high content in glutaminyl residues, the 11S and 7S seed storage proteins are rather poor substrates for this enzyme. This low reactivity has to be related to the low accessibility of the reactive residues due to the globular compact structure of these seed proteins. Chemical modifications such as succinylation (Lorand et al., 1971) or maleylation (Ikura et al., 1984) have been shown to open up globular proteins (β-lactoglobulin and lysozyme) to the transglutaminase. This phenomenon has not been explained in terms of structure modification.

In the present study, acylation was chosen as a way to modify the oligomeric conformation of the 11S seed globulins (Schwenke et al., 1990). The effect of alteration of substrate conformation—especially by increasing the degree of dissociation and unfolding—on the ability of TGase to modify a globular and oligomeric protein was

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investigated. The transfer and hydrolytic activites of TGase were estimated on pea legumin, which can be considered representative of the 11S-type seed proteins and because of its potential interest for food or nonfood applications. This protein has a relative molecular weight (M_r) of about 360 000 and is composed of six subunits ($\alpha\beta$) constituted of disulfide-linked acidic (α, M_r 40 000) and basic (β, M_r 20 000) polypeptides (Derbyshire et al., 1976; Casey, 1979). Citraconylation was used to modify its hexameric quaternary structure because of its potential reversibility (Brinegar and Kinsella, 1980).

MATERIALS AND METHODS

Preparation of Legumin. Legumin was prepared according to the method of Gueguen et al. (1984) with slight modifications as described by Larre and Gueguen (1986).

Citraconylation of Legumin. Legumin was dispersed in water at a concentration of 10 mg/mL. As described by Brinegar and Kinsella (1980), the citraconylation was performed at 28 °C by adding sufficient citraconic anhydride to obtain various degrees of acylation. The pH was maintained between 7.7 and 8.1 during the reaction by addition of 1 N NaOH. Excess of reagent was removed by dialysis for 24 h against water, and then the citraconylated protein was lyophilized.

The extent of lysyl residue modification was determined by the trinitrobenzenesulfonic acid (TNBS) method of Barber and Wharthesen (1982).

Ultracentrifugation. Ultracentrifugation of unmodified and modified legumin samples was performed at 4 °C for 20 h at 218000*g* in a 5–20% sucrose density gradient established in 0.1 M sodium phosphate buffer (pH 7.0) in a Beckman L5 65B type ultracentrifuge equipped with a swing-out rotor (type SW40 TI). An aliquot of 0.5 mL of protein solution (5 mg/mL) in 0.1 M sodium phosphate buffer (pH 7.0) was brought onto the top of the sucrose gradient. The dissociation of the protein into subunits was followed by evaluation of the sedimentation coefficients according to the method of Martin and Ames (1961).

Fluorescence Spectrophotometry. Fluorescence spectra were recorded at 25 °C using a SLM Instrument 4800 C fluorescence spectrophotometer. Protein samples were dissolved at 0.01 mg/mL in 0.1 M Tris-HCl (pH 7.5) and their spectra recorded in the range 300–400 nm with an excitation wavelength at 280 nm.

Purification of Guinea Pig Liver Transglutaminase (TGase). TGase was purified from fresh liver of guinea pig according to the method of Brookhart et al. (1983) with slight modifications. The livers were perfused with a 0.9% NaCl solution and then homogenized with a polytron homogenizer (Kinematica) in 25 mM sucrose solution containing 3 mM EDTA. The resulting homogenate was centrifuged at 40000*g* for 60 min; the supernatant was further clarified by ultracentrifugation at 170000*g* for 70 min (Beckman L5 65 B type ultracentrifuge rotor TI 70).

The crude enzymatic extract was applied on a column (2.5 × 20 cm) of DEAE-Sepharose CL-6B previously equilibrated with 5 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM benzamidine, and 0.15 M NaCl. The proteins were eluted with a linear gradient from 0.15 to 0.6 M NaCl at a flow rate of 100 mL/h. The active fractions were collected between 0.38 and 0.45 M NaCl and then purified on a column (2.5 × 20 cm) of hydroxyapatite (HA ultrogel IBF) previously equilibrated with 5 mM potassium phosphate (pH 7.2), 0.15 M KCl, 2 mM EDTA, 0.1 mM dithiothreitol (DTT), and 0.1 mM ATP. The proteins were eluted with a linear gradient from 5 to 200 mM potassium phosphate. The active fraction (1.5 units/mL) was obtained between 66 and 85 mM potassium phosphate, divided into small aliquots, and frozen at −20 °C until required.

TGase Activity Assay. TGase activity was measured according to the colorimetric hydroxamate procedure of Folk (1970). A unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of hydroxamate/min. Specific activity was expressed per milligram of protein, the concentration of which was estimated according to the method of Lowry et al. (1951).

Action of TGase on Legumin. Three different aspects of the action of TGase were followed both on native and on citraconylated legumin: deamidating efficiency of the enzyme; capacity to form glutamyl–lysyl cross-links; and ability to bind small amine molecules.

The enzymatic reaction was carried out at 37 °C in 0.06 M Tris-HCl (pH 7.5) containing 3 mM $CaCl_2$, 1.3 mM DTT, 1.35 mg/mL protein substrate, and TGase (0.5 unit/mL). The reaction was stopped by adding 20 mM EDTA, final concentration. The reaction mixtures were analyzed by HPLC gel filtration and SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Gel filtration analyses were performed on a Kontron HPLC System 400. The reaction mixture (100 μL) was injected into a column of Superose 12 HR (1.5 × 27.5 cm) previously equilibrated with 0.1 M Tris-HCl (pH 7.5). The flow rate was 0.4 mL/min, and the detection was performed at 280 nm.

The global enzymatic activity was followed by the amount of ammonia released, determined using glutamate dehydrogenase (EC 1.4.1.2) according to the method of Kun and Kearney (1974).

The degree of substrate cross-linking was followed by estimating the number of ε-(γ-glutamyl)lysyl bonds. The reaction products obtained after 4 h of incubation were dialyzed against water and lyophilized before analysis. Protein digests were obtained after sequential addition of proteolytic enzymes (subtilisin, pronase, leucine aminopeptidase, prolidase, and carboxypeptidase) according to the method of Griffin et al. (1982). Freeze-dried protein digests were derivatized by PITC (Bidlingmeyer et al., 1987). Amino acids and ε-(γ-glutamyl)lysine dipeptide were separated and quantified by RP-HPLC on a C_{18} column (Picotag Waters). Elution was achieved by a gradient from buffer A [0.07 M sodium acetate buffer (pH 6.6), 0.5 mL/L triethylamine] to buffer B (60% acetonitrile in buffer A) at 44 °C. Standards of amino acids as well as ε-(γ-glutamyl)lysine were used for the identification of the peaks and their quantitative evaluation.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out after reduction by mercaptoethanol of the TGase reaction products in homogeneous polyacrylamide (13%) slabs gels as described by Laemmli (1970). The gels were stained overnight with 0.1% Coomassie blue G250 in 25% methanol and 10% acetic acid. The destaining was performed in 5% acetic acid.

TGase efficiency for binding small amine molecules to different substrates was determined through the measurement of the amount of [^{14}C]putrescine incorporated into native and modified legumins using the method of Lorand et al. (1972) slightly modified. The reaction mixture (150 μL) containing 1 mg of the protein substrate and 36 mM putrescine was mixed with 200 nCi of [^{14}C]putrescine, 6.7 mM $CaCl_2$, 1.3 mM DTT in 0.1 M Tris-HCl (pH 7.5), and 0.5 unit of TGase. The enzymatic reaction was carried out at 37 °C for 4 and 24 h. After incubation, an aliquot of 60 μL of the reaction mixture was loaded onto a Whatman No. 3 paper disk of 22-mm diameter. This disk was immediately immersed in ice-cold 10% trichloroacetic acid to stop the enzymatic reaction by protein precipitation. Then it was washed twice in 5% trichloroacetic acid and once in acetone for 5 min and finally dried at 80 °C for 20 min. It was then placed in 3 mL of Ultima Gold scintillation cocktail and counted in a Packard Tri-Carb spectrometer for 10 min. All assays were made in duplicate. The experimental data were corrected from the nonspecific adsorption of the radiolabeled probe on the substrate and from the autoincorporation of this probe in the enzyme itself. These blank values corresponded respectively to 150–200 and 180 dpm. Consequently, the whole blank values subtracted from the experimental data were in the range 330–380 dpm. The TGase efficiency was expressed as nanomoles of putrescine incorporated into 1 mg of protein substrate. The respective incorporation of [^{14}C]putrescine into the polymerized and nonpolymerized forms of the reaction products was determined from the bands separated by SDS-PAGE.

RESULTS AND DISCUSSION

Substrates Characterization. To avoid the participation of the protein amino groups in the transfer reaction, they were blocked by citraconylation. The acylation of

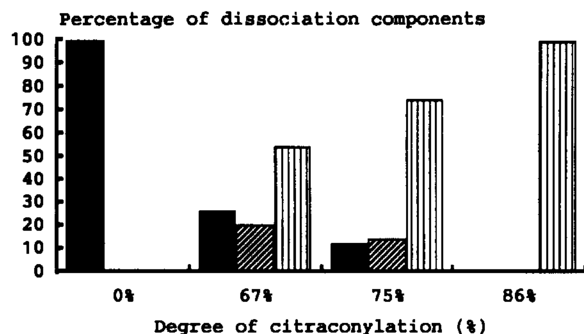

Figure 1. Dependence of the percentage of the legumin dissociation components on the degree of citraconylation: (solid bars) 12S; (slashed bars) 7S; (striped bars) 3S.

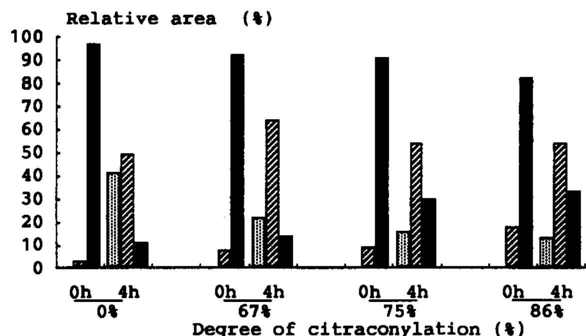

Figure 2. Evaluation of the apparent molecular weight by HPLC gel filtration of the substrates and their products obtained after 4 h of TGase reaction. Reaction conditions: 0.06 M Tris-HCl (pH 7.5) containing 3 mM $CaCl_2$, 1.3 mM DTT, 1.35 mg/mL protein substrate, and 0.05 unit/mg TGase at 37 °C. Elution conditions: 0.1 M Tris-HCl (pH 7.5); flow rate, 0.4 mL/min. (Solid bars) 360 000; (slashed bars) 720 000; (dotted bars) 2 000 000.

lysyl residues performed at pH 8 with anhydride/lysine molar ratios of 7 and 14 led to 67% and 75% of lysyl residues modified. After freeze-drying, the 75% acylated legumin was treated again with a 14-fold molar excess of anhydride. A degree of 86% citraconylation was reached. Citraconylation by increasing negative charges induced a stepwise dissociation of the 12.5S sedimenting native legumin into 7.4S and 3.3S components as shown by ultracentrifugation (Figure 1). For the 67% and 75% modified legumins, the 3S form was already predominant but 12S and 7S components still remained. When the lysyl acylation reached 86%, only 3S components were present.

As for succinylated 11S-type proteins, this dissociation occurred gradually and the three forms were found together at intermediate levels of citraconylation (Schwenke et al., 1985, 1990; Shetty and Rao, 1978). It can be expected that the 12S hexameric form $(\alpha\beta)_6$ should dissociate into 7S trimeric forms $(\alpha\beta)_3$. Then in a second step, dissociation into monomers corresponding to the 3S unfolded polypeptides $(\alpha\beta)$ should occur.

For each protein, the HPLC gel filtration pattern revealed a main peak corresponding to an apparent molecular weight of 360×10^3, which became asymmetrical for the more citraconylated legumins. This shape change has to be related to the appearance of high hydrodynamic components resulting from the chemical modification. To quantify these forms, each chromatogram was divided into three sections corresponding to an average molecular weight of 720×10^3 and 360×10^3. As shown by Figure 2 the 720×10^3 section relative area increased from 3% for the unmodified legumin to 17% for the most citraconylated protein (86%). The presence of a main peak at 360×10^3 for each level of citraconylation indicates that

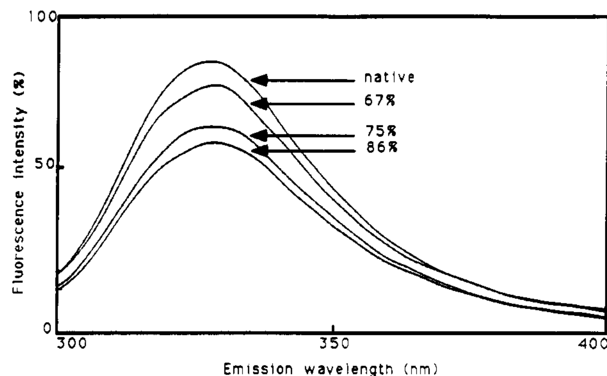

Figure 3. Fluorescence emission spectra of legumin at various levels of citraconylation in 0.1 M Tris-HCl (pH 7.5). Excitation wavelength was 280 nm.

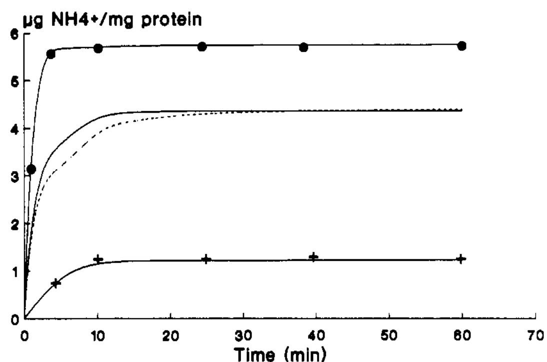

Figure 4. Rate of ammonia production during TGase reaction with various legumins. Experimental conditions: 0.06 M Tris-HCl (pH 7.5) containing 3 mM $CaCl_2$, 1.3 mM DTT, 1.35 mg/mL protein substrate, and 0.05 unit/mg TGase at 37 °C. (+) native legumin; (- - -) 67% citraconylated; (—) 75% citraconylated; (●) 86% citraconylated.

the different dissociated forms (7S, 3S) have hydrodynamic volumes similar to that of the globular native one (12S) and consequently that the 7S and 3S forms are certainly rather unfolded.

Moreover, the fluorescence intensity level observed in the emission spectrum of citraconylated legumins is lower than that observed for the native protein (Figure 3). This gave evidence for the alteration of the three-dimensional structure of legumin by citraconylation. Schwenke et al. (1990) pointed out the same effect on legumin succinylated at levels up to 75% and noted moreover a shift of the maximum fluorescence intensity.

Action of TGase on Native and Citraconylated Legumin. Native or modified legumins were compared either as single substrate for transglutaminase or in the presence of a low molecular weight amine molecule as second substrate.

Single Substrate Conditions. The measurement of total ammonia released during the TGase reaction provided good information about the substrate reactivity. Kinetics obtained for native and citraconylated legumins are reported Figure 4; in all cases the liberation of ammonia increased in the first 20 min of reaction and then leveled out. The total ammonia liberated was lower for native legumin (1 µg of NH_3/mg of protein) than for citraconylated substrates (4.2–5.6 µg of NH_3/mg of protein). It did not differ significantly for the 65% and 75% citraconylated legumins. The extents of ammonia liberated after 4 h are expressed in moles per mole of protein (Table I) and compared for various levels of acylation.

As the modified legumins were only partially citraconylated, the participation of the nonblocked amine groups

Table I. Comparison of Unmodified and Modified Legumins as Substrates for TGase[a]

	NH_3 liberated,[b] mol/mol	Glu–Lys bond,[b] mol/mol	putrescine incorporated,[c] mol/mol
native legumin	21	13	25
67% acylated legumin	42	10	51
75% acylated legumin	52	9	58
86% acylated legumin	105	4	89

[a] Results expressed on the basis of an average molecular weight of 360 × 10^3 for native and modified legumins. [b] Experimental conditions: measurement of total NH_3 liberated and Glu–Lys bonds formed after 4 h of incubation of only one substrate with TGase in 0.06 M Tris-HCl (pH 7.5) containing 3 mM $CaCl_2$ and 1.3 mM DTT at 37 °C. [c] Putrescine incorporation was measured after 4 h of incubation of the protein substrate (1 mg) and 36 mM putrescine was mixed with 200 nCi of [^{14}C]putrescine in 0.1 M Tris-HCl (pH 7.5) containing 6.7 mM $CaCl_2$ and 1.3 mM DTT at 37 °C.

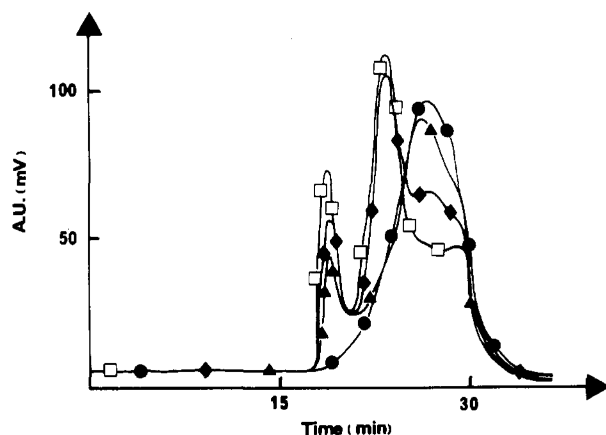

Figure 5. Evolution of the HPLC gel filtration patterns during the TGase reaction on the legumin citraconylated at level 75%. Experimental conditions: 0.06 M Tris-HCl (pH 7.5) containing 3 mM $CaCl_2$, 1.3 mM DTT, 1.35 mg/mL protein substrate, and 0.05 unit/mg TGase at 37 °C. (●) $t = 0$ min; (▲) $t = 10$ min; (◆) $t = 30$ min; (□) $t = 120$ min and more.

in the transfer reaction was investigated, first by HPLC and SDS electrophoresis and then by measuring the number of ε-(γ-glutamyl)lysyl bonds.

HPLC gel filtration of the TGase reaction products showed very similar profiles whatever was the substrate, modified or not. As seen in Figure 5, when the 75% citraconylated sample is treated by TGase, the fraction corresponding to 360 × 10^3 decreased while two fractions corresponding to apparent molecular weights of 720 × 10^3 and up to 2 × 10^6 appeared. Moreover, in each case the reaction was completed within 1–4 h. The products obtained after 4 h of reaction with the modified and unmodified legumin were compared by HPLC gel filtration. Each chromatogram was divided into three sections (2 × 10^6, 720 × 10^3, 360 × 10^3) as previously described and their relative areas in the case of native and citraconylated legumin products are reported in Figure 3. The proportion of high apparent molecular weight polymers formation was higher for native legumin than for citraconylated forms. For each substrate the peak corresponding to 360 × 10^3 apparent molecular weight was still present after incubation. Consistent results were obtained by SDS–PAGE in reductive conditions. A part of the reaction products did not even penetrate the gel, indicating the presence of polymers covalently cross-linked (results not shown).

A quantitative analysis of the rate of cross-linking was obtained by assessment of the ε-(γ-glutamyl)lysine dipeptides isolated after proteolytic degradation of the TGase-modified proteins. The results are summarized in Table I. As expected and in agreement with the HPLC results, the extent of glutamyllysine dipeptide decreased when the level of acylation increased.

Two-Substrate Conditions. In a second part, the ability of the various legumin samples to be substituted by low molecular weight primary amine was tested by using [^{14}C]-putrescine as second substrate. After 4 h of incubation, the incorporation of putrescine into native legumin reached 25 mol/mol. It drastically increased from 50 to 89 mol/mol when the acylation level was raised from 67% to 86%, respectively (Table I).

Two of these products, obtained with native legumin and with legumin citraconylated at 86% as substrates, were subjected to SDS–PAGE. The electrophoretic patterns revealed the presence of large molecules which were unable to penetrate the gel for both substrates, and radiolabeled putrescine was detected in their polymeric fractions. This incorporation of [^{14}C]putrescine into the polymerized fraction of the product indicated that polymerization and amine incorporation occurred at the same time.

Influence of the Substrate Conformation. As far as amine incorporation or deamidation by TGase is concerned, native legumin was shown to be a rather poor substrate but its reactivity can be increased by citraconylation. These observations are consistent with those of Lorand et al. (1971) and of Ikura et al. (1984), who observed a positive effect of succinylation in the case of lysozyme and β-lactoglobulin and of maleylation in the case of bovine serum albumin and ovalbumin. It could be related to conformational changes which led to a greater availability of the glutaminyl residues as donors in the TGase-catalyzed incorporation of amino compounds. The change in the reactivity of legumin depending on the degree of citraconylation will be discussed, taking into account the modifications of its oligomeric structure.

The complete structure of the legumin has not been yet elucidated, but two models based on the $(\alpha\beta)_6$ oligomeric structure are generally considered (Badley et al., 1975; Plietz et al., 1984). According to the model of Plietz et al. (1984), the α hydrophilic polypeptide is mainly located at the periphery of the protein. From the 37 glutaminyl and 22 lysyl total residues of the (αβ) subunit, about 33 glutaminyl and 13 lysyl residues are located in hydrophilic regions of the sequence and should be therefore situated at the surface of the protein. Moreover, on the basis of the model of Plietz and according to chemical modifications results, Gueguen (1990) estimated that only 16 glutaminyl and 11 lysyl residues could be effectively accessible.

From the results obtained on native legumin (Table I), it appeared that two glutaminyl residues per (αβ) subunit formed ε-(γ-glutamyl)lysyl bonds while one or two were hydrolyzed into glutamyl residues. Reactive glutamine represented only, respectively, 9% of the total amount and 21% of the presumed accessible one. The same calculations for lysine gave almost the same level of reactivity: 9% of the total amount of lysine and 18% of the presumed accessible lysine. This low reactivity had to be related to the globular conformation of the protein and to the vicinity of glutaminyl residues.

Citraconylation significantly increased the reactivity of legumin in the TGase reaction. The level of deamidation rose from 9% for the native legumin to 45% for a 86% citraconylated one. Only half of the glutamyl residues became available, even when the protein was completely unfolded. This partial deamidation can be related to the known influence of adjacent amino acid residues on the reactivity of amide groups. It may result not only from

the legumin primary sequence but also from steric and charge effects due to the introduction of citraconyl groups.

A partial citraconylation only limited the formation of protein–protein conjugates when no external amine was added. When lysines were blocked at a level of 86%, less than one cross-link per subunit was measured.

When putrescine was used as an acyl acceptor, only 40% of the total glutaminyl residues acted as γ-carboxamide group donors. The reactivity level of the amide groups for amine incorporation was slightly lower than that obtained without acyl acceptor for deamidation. This suggested that the former putrescine bound to the protein could restrain the accessibility of the glutaminyl residues situated in a close vicinity. This can support the proposal of Mycek and Waelsch (1960) that the same sites may be involved in both the replacement and hydrolysis reactions.

Conclusion. The introduction of charges on the legumin by means of citraconylation induced conformational changes, dissociation and unfolding, which modified accessibility of glutaminyl and unblocked lysyl residues. Citraconylation is known to be chemically reversible; in their work on α_{s1}-casein Motoki et al. (1987) showed that after decitraconylation, the protein recovered a structure close to the native one. They can therefore study the single influence of the enzymatic modification on the physicochemical characteristics of the protein. In the case of legumin the decitraconylation led to a structure completely different from the native one (unpublished results). It became therefore difficult to separate the citraconylation–decitraconylation effect from that of enzymatic modifications in terms of structure, physicochemical properties, and food functionality as it was proposed (Motoki et al., 1986) on α_{s1}-casein.

This study showed the usefulness of this technique to convert 11S seed globulins into good substrates for TGase and to enhance deamidation. More generally, it can be concluded that unfolded proteins and polypeptides act as better substrates than globular ones. Work is now planned to reach the protein unfolding by nonchemical treatments to avoid the acylation effect. As deamidation, amine incorporation, and cross-links proceed in parallel, further investigations are also needed to control the whole reaction.

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