Preparation and Physicochemical Properties of Glycosylated Derivatives of Pea Legumin

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Neoglycolegumins were prepared by reductive alkylation of amino groups of pea legumin with various carbohydrates. The percentage of modification of amino groups was 84% with galactose, 59% with glucose, 49% with lactose, and 18% with galacturonic acid. Electrophoretic behavior and sedimentation studies showed that the glycosylation of legumin tends to decrease the aggregated forms observed for unmodified legumin, while partially dissociated forms appeared especially for legumin modified with galacturonic acid. The kinetics of glycosylation and the electrophoretic patterns indicated that all the amino groups did not react at the same rate. The higher glycosylation rate observed for the α-polypeptides suggests that they are more exposed to the protein surface than the β-polypeptides. Analyses of ultraviolet difference and circular dichroism spectra showed that, among the 35 nonaccessible tyrosyl residues in the unmodified legumin, only 4–7 residues became unburied after glycosylation. It is probable that the inner part of the neoglycoprotein keeps its close packed structure.

INTRODUCTION

In past years, great interest has been focused on plant proteins as a source of food proteins. However, their potential utilization in foodstuffs seems to depend in large part on their functional properties. These are derived from physicochemical characteristics such as molecular weight, amino acid composition and sequence, conformation, net surface charge, and effective hydrophobicity (Kester and Richardson, 1984). Any modification capable of altering one or more of these characteristics may also change one or several functional properties.

Glycosylation of proteins by attachment of hydrophilic groups is one potential means of modifying their characteristics. A number of chemical methods for the covalent attachment of sugar residues to proteins have been developed [for a general review see Stowell and Lee (1980)]. Neoglycoproteins have been prepared to investigate the contribution of the carbohydrate moiety to the immunological and biological functions mediated by glycoproteins (Nixdorff et al., 1975; Krantz et al., 1976; Kamicker et al., 1977). They have also been used as artificial antigens for the study of anti-carbohydrate antibodies (Arakatsu et al., 1966; Lemieux et al., 1977), as potential substrates for lectins (Iyer and Goldstein, 1973; Goldstein et al., 1975; Lönngren et al., 1976) and glycoprotein clearance (Marsh et al., 1977), and as affinity materials for chromatography (Stowell and Lee, 1978). In other cases, polysaccharide–enzyme conjugates have been synthesized to enhance the stability of enzymes to heat and proteolysis (Marshall and Rabinowitz, 1975, 1976; Marshall, 1976; Marsh et al., 1977). A number of these conjugates may have potential as therapeutic agents (Marshall, 1978).

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In spite of the intensive research on a variety of proteins, attempts to glycosylate food proteins are rather limited. Some studies have been performed on albumin (Gray, 1974; Schwartz and Gray, 1977; Lee and Lee, 1980), β-lactoglobulin (Waniska and Kinsella, 1984a,b; Kitabatake et al., 1985), and casein (Lee et al., 1979; Canton and Mulvihill, 1983; Courthaudon et al., 1989). A better solubility of these carbohydrate-derivatized proteins was generally emphasized. To our knowledge, no work was undertaken with vegetable proteins. The latter generally exhibit a relatively poor solubility and as a result are difficult to use at high concentrations. For the purposes of studying and of improving the functional properties of 11S-type seed globulins as well as of establishing the relationships which exist between these properties and the structural and physicochemical characteristics, we have recently developed a large-scale purification of legumin (Gueguen et al., 1984; Larre and Gueguen, 1986), the 11S storage protein of pea (*Pisum sativum*) seeds. Legumin with a relative molecular weight (M_r) of about 360 000 is a good substrate representative of seed proteins of 11S type. It is composed of six ($\alpha\beta$) subunit pairs, each of which consists of disulfide-linked acidic (α, M_r 40 000) and basic (β, M_r 20 000) polypeptides (Derbyshire et al., 1976; Casey, 1979).

The present study reports the covalent attachment of glycosyl residues (galactose, glucose, lactose, and galacturonic acid) to legumin by alkylation and determines the resulting effects on some physicochemical properties of the modified legumin. Under the experimental conditions, the acyclic form of carbohydrates reacts reversibly and selectively with free amino groups of proteins to yield unstable Schiff bases which can be stabilized in vitro by reduction with sodium cyanoborohydride. In vivo, the nonenzymatic incorporation of glucose occurs into proteins including lens cristallins, insulin, basic myelin protein, erythrocyte membrane proteins, collagen, albumin, immunoglobulins, and hemoglobin [for a recent review, see Kennedy and Lyons (1989)]. For these biological proteins, the Schiff base can undergo in vivo the Amadori

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rearrangement to afford a stable ketoamine derivative which then cyclizes to the hemiketal structure.

MATERIALS AND METHODS

Materials and Chemicals. Legumin was purified from pea flour (*P. sativum*, variety Amino) by a chromatographic procedure using successive ion-exchange and gel filtration steps as described previously (Gueguen et al., 1984; Larre and Gueguen, 1986). Sodium cyanoborohydride and 2,4,6-trinitrobenzenesulfonic acid were purchased from Aldrich Chemical Co. Carbohydrates and galacturonic acid were obtained from Merck. D-[U-^{14}C]galactose (210 mCi/mmol, i.e., 7.77 GBq/mmol) was from CEA (Commissariat à l'Energie Atomique, Gif/Yvette). All other reagents and chemicals were of analytical grade.

Glycosylation of Legumin by Reductive Alkylation. A modification of the procedure of Gray (1974) was used. In a typical experiment, the reaction was carried out at 37 °C in 9 mL of 0.12 M sodium carbonate (pH 8.5) containing legumin (0.2 μmol), sodium cyanoborohydride (5.28 mmol), and various amounts of carbohydrate or galacturonic acid. The progress of the reaction was monitored by withdrawing aliquots at different times (between 1 and 120 h). Finally, the solutions were dialyzed at 4 °C against 0.12 M sodium carbonate (pH 8.5) for 48 h with five changes of the buffer. When the reaction was performed with [^{14}C]galactose, the specific radioactivity was adjusted to 0.02 μCi/μmol galactose.

Determination of the Extent of Glycosylation. The amount of lactose coupled to legumin by reductive amination could be determined by the phenol–sulfuric acid method (Dubois et al., 1956) using galactose as the standard for the calibration curve; indeed, since the secondary amine formed by reductive amination is acid stable, only the glycosidically linked galactosyl residue is removed during the hydrolysis and determined by this method. For the same reasons, monosaccharides and galacturonic acid directly coupled to protein by reductive amination could not be determined by the phenol–sulfuric acid method. These carbohydrates as well as lactose attached to legumin by ketoamine linkage were measured by the method of Hantzsch (Nash, 1953), as modified by Gallop et al. (1981).

The radiolabeled galactose covalently bound to legumin was determined from dialyzed aliquots of 0.5 mL diluted with 0.5 mL of Soluene 350 (Packard). Radioactivity was counted after 10 mL of Picofluor 40 (Packard) was added in a Packard 1500 Tri-Carb liquid scintillation counter.

Determination of Protein Concentration and of the Number of Unsubstituted Amino Groups. Protein concentrations were determined by the method of Lowry et al. (1951) using unmodified legumin as a standard or from UV absorbance with $E_{1\,\mathrm{cm}}^{1\%}$ of 6.0. The number of free (unsubstituted) amino groups in legumin samples was determined either by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described by Fields (1972) or by the Moore and Stein method (1954).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970) on 0.75 mm thick slab gels (7 cm × 8 cm). The running gel contained 14% (w/v) acrylamide and the stacking gel 5% acrylamide. Protein bands were stained with Coomassie brillant blue R250. Prior to analysis, the protein samples were denatured by a 4-min treatment at 100 °C in a 50 mM Tris-HCl buffer (pH 6.8) containing 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 15% (v/v) glycerol, and 0.025% (w/v) bromophenol blue. Electrophoresis of the undenatured legumin samples was also performed but with running gels of 8% (w/v) in acrylamide without SDS.

Ultraviolet Spectroscopy. The degree of exposure of the tyrosyl residues (y), depending on the level of glycosylation and the nature of carbohydrate, was provided by the second-derivative spectra of the protein. According to Ragone et al. (1984), it is defined as

$$y = (R_n - R_a)/(R_u - R_a)$$

and expressed as the percentage of the total tyrosyl residues in the protein. R_n and R_u were obtained by the ratio

$$(A''_{\lambda 1} - A''_{\lambda 2})/(A''_{\lambda 3} - A''_{\lambda 4})$$

calculated for the native protein (R_n), in a 0.1 M phosphate buffer (pH 8), and in its unfolded conformation (R_u), in 6 M guanidine hydrochloride. In the case of a tyrosine–tryptophan mixture, the theoretical values for λ1, λ2, λ3, and λ4 were 287, 283, 295, and 290.5 nm, respectively. These values were slightly shifted for the protein samples (Figure 1). In all cases, the values of R_n and R_u were calculated by using the maxima of the corresponding peaks. R_a would correspond to the above ratio for a tyrosine–tryptophan mixture at a molar ratio x, identical with that of legumin, the mixture being dissolved in a buffer having the same characteristics as the interior of the protein. A value of −0.17 was found for R_a from the expression established by Ragone et al. (1984):

$$R_a = (Ax + B)/(Cx + 1)$$

In this expression, a value of 4.33 was used for x as calculated from the sequence of the legumin αβ-subunit determined by Lycett et al. (1984), assuming that this theoretical value is close to the exact one; it is indeed known that legumin is not composed of six identical αβ subunits. The constants A, B, and C were those determined by Ragone et al. (1984) (i.e., A = −0.18, B = 0.64, C = −0.04).

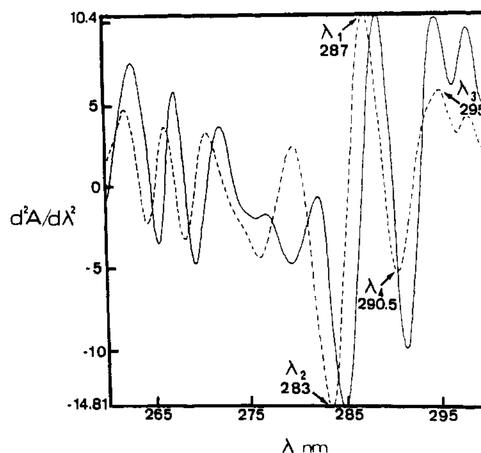

Figure 1. Second-derivative UV spectra in 0.1 M phosphate buffer, pH 8.0. (- - -) *N*-Acetyl-L-tyrosine ethyl ester plus *N*-acetyl-L-tryptophan ethyl ester mixture (Tyr/Trp molar ratio, 4.33); (—) galactosylated legumin (degree of modification of amino groups, 19%).

Circular Dichroism. The ultraviolet circular dichroism spectra were established in the range 190–260 nm at room temperature by using a Jobin Yvon Mark 5 dichrograph equipped with 0.1 mm path length quartz cells and linked to a microcomputer. The UV–CD spectra were the average of 40 measurements for each sample. The protein concentration was about 1 mg/mL.

The proportions of α-helix, β-sheets, and random coil segments in the protein were estimated by fitting the experimental CD spectra as the sum of published reference spectra. The fitting was performed by the method of Chen et al. (1972, 1974) or Brahms and Brahms (1980) using a general least-squares program. The random coil regions were considered as the regions without α-helix and β-sheets. They included the regions described as aperiodic or having irregular secondary structures.

Ultracentrifugation. Centrifugation was performed in 5–20% (w/v) linear isokinetic sucrose gradients prepared with a 0.1 M phosphate buffer (pH 8). Protein samples (0.25 mL at 5 mg/mL) were layered at the top of the gradients and centrifuged for 18 h at 285000*g* and 20 °C by using a Beckman L5 65B centrifuge equipped with a SW 40 Ti rotor.

RESULTS AND DISCUSSION

Kinetics of Coupling of Glycosyl Residues to Legumin. The influence of reaction time on the extent of glycosylation of legumin by reductive alkylation was

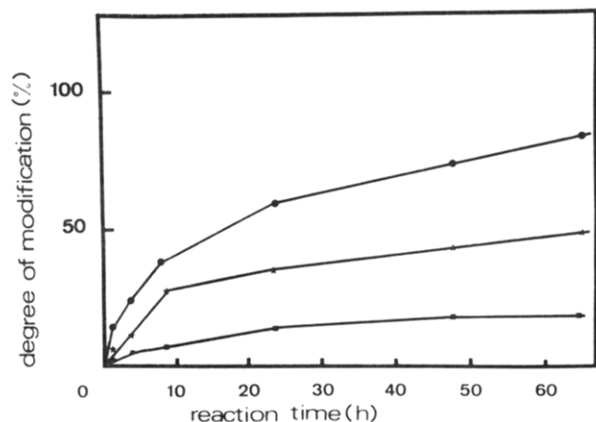

Figure 2. Kinetics of glycosylation of legumin performed at 37 °C with galactose (●), lactose (★), and galacturonic acid (■) in the presence of cyanoborohydride in 0.12 M sodium carbonate buffer (pH 8.5).

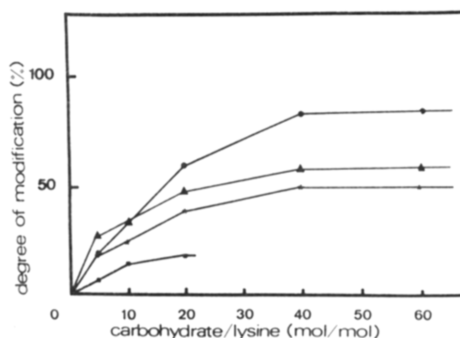

Figure 3. Effect of the carbohydrate/lysine groups (mol/mol) ratio on the degree of glycosylation of legumin. The reaction was performed at 37 °C for 65 h with galactose (●), glucose (▲), lactose (★), and galacturonic acid (■).

studied with a large excess of carbohydrate in the reaction medium buffered at pH 8.5, where the cyanoborohydride anion selectively reduces the Schiff base formed between the carbonyl groups of reducing sugars and protein-free amino groups (Borch et al., 1971). Since it has been shown that the hydroxyl groups could react with tyrosine side chains (Friedman et al., 1982), we have verified, by amino acid analysis, that only the amino groups were effectively modified by glycosylation under our experimental conditions. Both the number of carbohydrate residues covalently bound to legumin and the number of remaining amino groups were determined to evaluate the progress of the reaction. The different methods determining the degree of glycosylation gave concordant results. These were directly correlated with the number of unsubstituted amino groups as determined by the method of Moore and Stein (1954). On the contrary, the results based on the TNBS analyses were not related to the results obtained by the other methods. Considering the complex structure of legumin, it is probable that the glycosylation leads to a modification of the protein structure and unmasks lysyl residues inaccessible to TNBS in the native molecule. As shown in Figure 2, whatever the carbohydrate coupled to legumin, the level of modification reached an almost constant value after 65 h of reaction.

The effect of carbohydrate concentration in the reaction medium was determined with carbohydrate/amino groups molar ratios varying in a very wide range (Figure 3). For galactose, glucose, and lactose, an optimal coupling was obtained by using a 60-fold molar excess. In the case of galacturonic acid, above a 20-fold molar excess, a

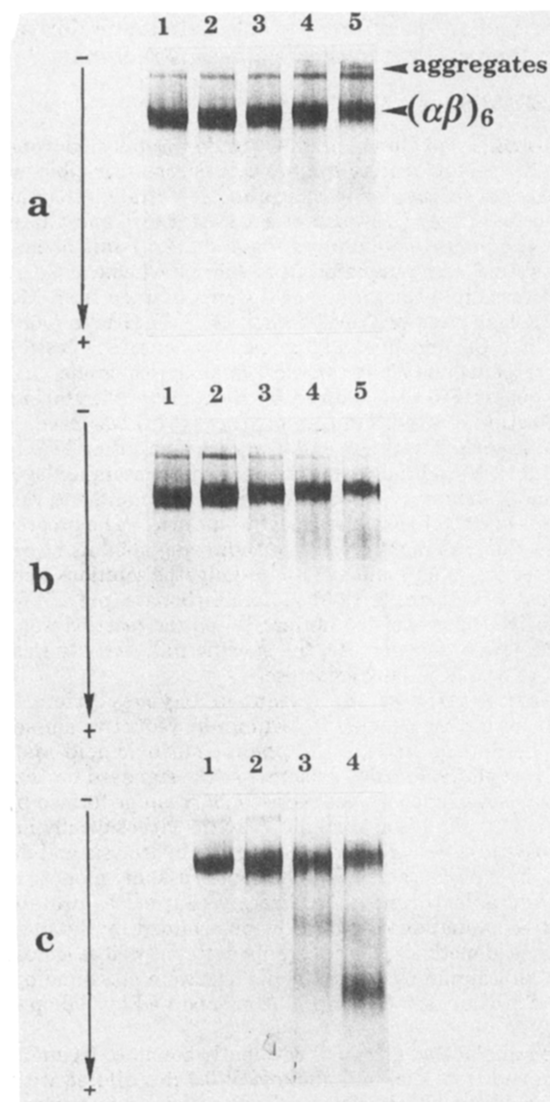

Figure 4. Polyacrylamide gel electrophoresis of control and glycosylated legumin. (a) Galactosylated derivatives at various degrees of modification of amino groups: (lane 1) 0%; (lane 2) 19%; (lane 3) 34%; (lane 4) 61%; (lane 5) 84%. (b) Lactosylated derivatives: (lane 1) 0%; (lane 2) 20%; (lane 3) 26%; (lane 4) 39%; (lane 5) 49%. (c) Galacturonic acid derivatives: (lane 1) 0%; (lane 2) 7%; (lane 3) 15%; (lane 4) 18%.

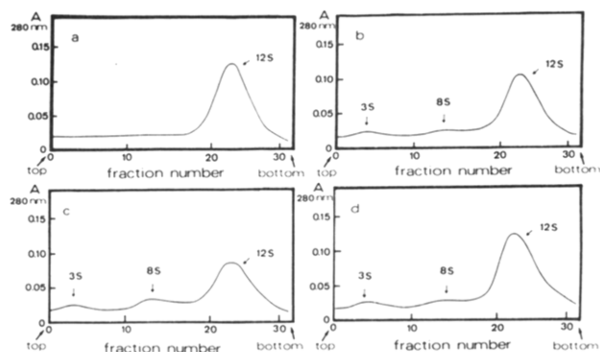

Figure 5. Sedimentation profiles of control and glycosylated legumin. (a) Unmodified legumin; (b) galactosylated derivative (degree of modification of amino groups; 33%); (c) lactosylated derivative (16%); (d) galacturonic acid derivative (7%).

precipitation of legumin was observed, probably due to ionic interactions (Schwenke et al., 1988).

The efficiency of the reaction was also dependent on the nature of the carbohydrate. Figure 3 shows that, under the optimal conditions described above, the percentage of

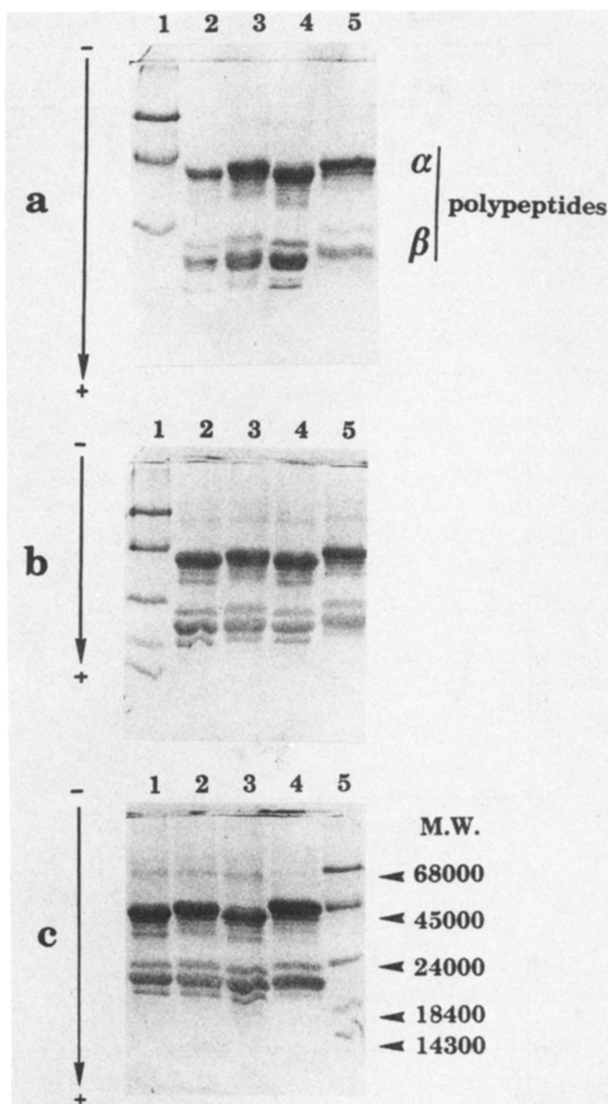

Figure 6. SDS–polyacrylamide gel electrophoresis of control and glycosylated legumin. (a) Galactosylated derivatives at various degrees of modification of amino groups: (lane 1) molecular weight standards; (lanes 2 and 4) 0%; (lane 3) 34%; (lane 5) 84%. (b) Lactosylated derivatives: (lane 1) molecular weight standards; (lanes 2 and 4) 0%; (lane 3) 26%; (lane 5) 49%. (c) Galacturonic acid derivatives: (lanes 1 and 3) 0%; (lane 2) 7%; (lane 4) 18%; (lane 5) molecular weight standards.

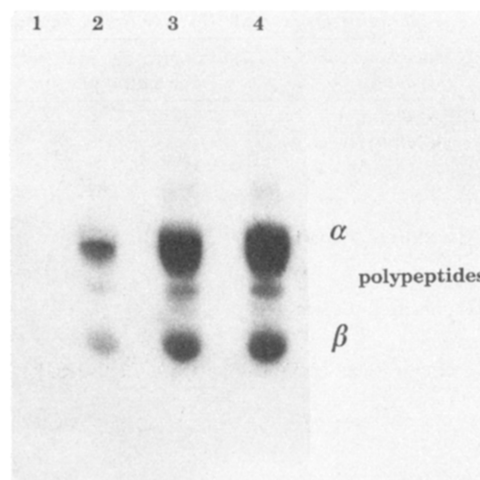

Figure 7. Autoradiography of SDS–polyacrylamide gel electrophoresis performed with [^{14}C]galactosylated legumin at various degrees of modification of amino groups: (lane 1) 0%; (lane 2) 27%; (lane 3) 39%; (lane 4) 61%.

modification of amino groups in the legumin was 84% with galactose, 59% with glucose, 49% with lactose, and 18% with galacturonic acid. These results are in good agreement with those reported in the literature for animal proteins modified by the same procedure. Lee et al. (1979) and Courthaudon et al. (1989) found that monosaccharides are better substrates than disaccharides for reductive alkylation of caseins. Urbanowski et al. (1982) reported that, in vitro, galactose glycosylated human serum albumin both faster and more extensively than glucose under various alkylation conditions.

Electrophoretic Behavior and Sedimentation Characteristics of Glycosylated Legumin Derivatives. Under nondenaturing conditions, the unmodified legumin displayed in polyacrylamide gel electrophoresis (PAGE) major and minor bands which correspond respectively to the $(\alpha\beta)_6$ native conformation and to aggregates (Figure 4). In the case of the glycosylated legumin, this pattern was slightly modified according to the nature of the carbohydrate and the extent of modification. The main band did not move as far as the unmodified legumin when the glycosylation degree increased. This effect was probably due to an increase of the hydrodynamic volume of the molecule. The band corresponding to aggregates generally tended to decrease in intensity with the extent of glycosylation and completely disappeared for the highest levels of modification with lactose and galacturonic acid (Figure 4b,c). For legumin modified with galactose (Figure 4a), aggregates still remained present. For the highly galactosylated derivatives, a new band, corresponding to aggregates of higher molecular weights, even appeared.

In addition, whatever the glycosylated derivative, new bands with a faster migration appeared for the high levels of modification (Figure 4), indicating a partial dissociation into subunits of the legumin. This phenomenon is particularly pronounced with galacturonic acid derivatized legumin samples (Figure 4c) for which the dissociated components moved faster than those observed with galactosylated or lactosylated legumin (Figure 4a,b), owing to the charge brought by the galacturonic acid molecules. These results were corroborated by ultracentrifugation assays. As shown in Figure 5, components with sedimentation coefficients of about 8S and 3S appeared for glycosylated samples. According to our previous results (Gueguen et al., 1988), these dissociated components should correspond respectively to $(\alpha\beta)_3$ and $\alpha\beta$ forms assuming the commonly adopted $(\alpha\beta)_6$ oligomeric structure for the unmodified legumin.

Under denaturing conditions, electrophoresis (SDS–PAGE) allowed the progress of the glycosylation reaction toward each family of α and β constitutive polypeptides (Figure 6) to be followed. The autoradiography performed after electrophoresis of the legumin treated with [^{14}C]-galactose showed that both types of polypeptides (α and β) were modified (Figure 7).

An approximation evaluation of the amount of carbohydrates bound to the major α- and β-polypeptides was carried out on the basis of SDS–PAGE pattern by measuring the increase of their molecular weight due to glycosylation. These results were compared to the content of the amino groups in the α (14 amino groups) and β (10 amino groups) polypeptides. These values were chosen according to the sequence published by Lycett et al. (1984), although differences exist between the various α- and β-polypeptides. After a reaction time of 24 h under our experimental conditions, the degree of galactosylation reached values close to 100%, for the two α-poly-

Table I. Effects of Degree of Glycosylation on the Structure of Legumin

protein	degree of modification of amino groups, %	no. of exposed tyrosyl residues[a]	α-helix,[b] %	β-sheets,[b] %	random coil,[b] %
unmodified legumin	0	43	9	46	45
galactosylated derivatives	19	47	21	21	58
	34	47	21	22	57
	61	45	22	22	56
	84	49	21	18	61
lactosylated derivatives	20	48	19	22	58
	39	49	19	21	60
	49	50	18	19	63
galacturonic acid derivatives	7	43	23	24	53
	14	45	20	22	58

[a] Determined from second-derivative UV spectra. [b] Determined from circular dichroism spectra.

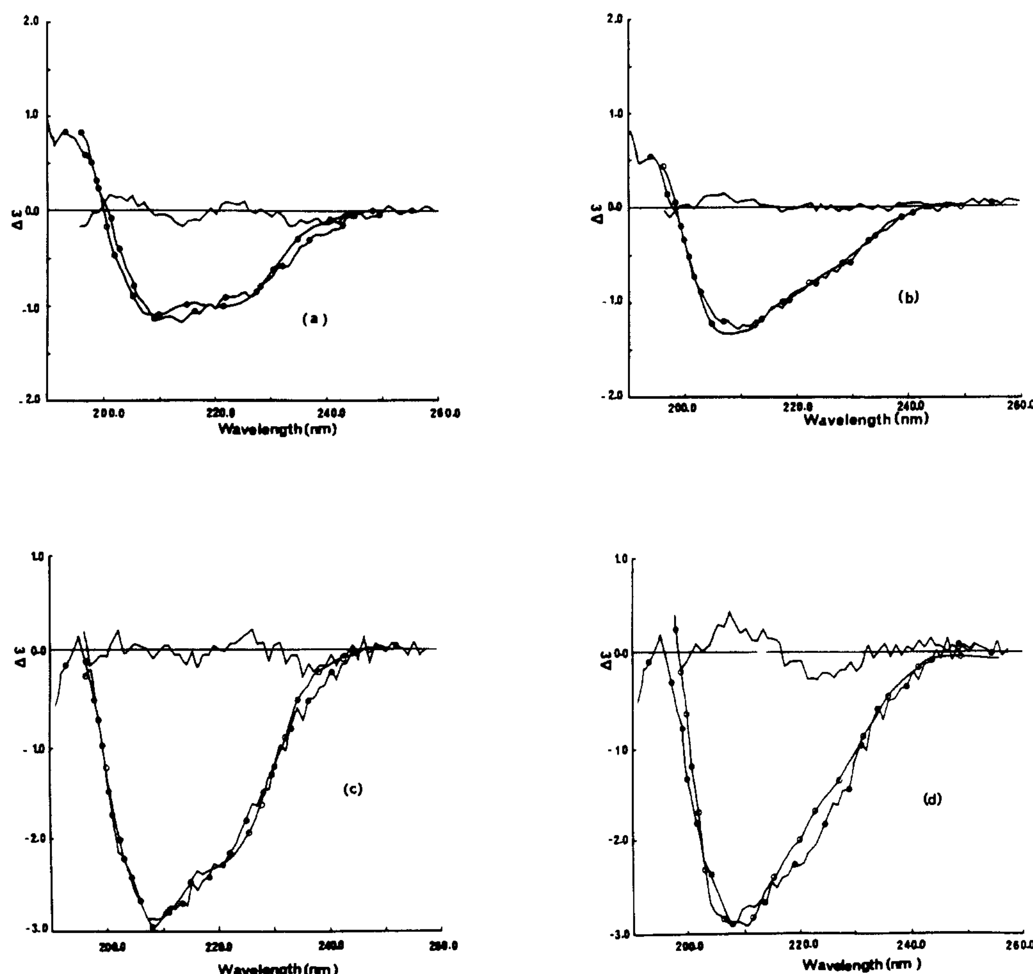

Figure 8. Ultraviolet circular dichroism spectra of control and glycosylated legumin. (a) Unmodified legumin spectra fitted by the method of Chen; (b) unmodified legumin spectra fitted by the method of Brahms; (c) galactosylated derivative spectra fitted by the method of Chen; (d) galactosylated derivative spectra fitted by the method of Brahms. (●) Experimental spectra; (O) calculated spectra; (—) difference between experimental and calculated spectra.

peptides studied, and only 50% for the β-polypeptides. Comparing the levels of maximum glycosylation revealed that the number of glycosylated amino groups reached the highest value for galactose and was lower for lactose and galacturonic acid. These differences, which are in good agreement with the kinetic curves, showed that some of the amino groups were only accessible for galactose and could not be reached by the two other carbohydrates, probably due to either their charge or their size.

Moreover, the higher glycosylation rate generally obtained for the α-polypeptides should signify that they were more exposed to the protein surface than the β-polypeptides.

Analyses of Ultraviolet Difference and Circular Dichroism (CD) Spectra. By use of the second-derivative UV spectra, the degree of tyrosyl residue exposure (y) was evaluated as a function of the glycosylation level and the type of carbohydrate bound. As shown in Table I, about 43 tyrosyl residues (of the 78 total ones) are exposed to the surface of the unmodified legumin in a 0.1 M phosphate buffer (pH 8). This result was in good agreement with the 40 tyrosyl residues found in a previous study at pH 7.0 (Gueguen et al., 1988). The accessibility of these chromophores was generally slightly increased by glycosylation, but seemed to be only little dependent on the alkylation degree. The number of tyrosyl residues exposed was about of 47–49, 48–50, and 43–45 for galactosylated, lactosylated, and galacturonic acid derivatives, respectively. It seems that the binding of mono- and disaccharides to legumin induces a slight opening of the quaternary and

tertiary structures. However, considering that among the 35 nonaccessible tyrosyl residues in the unmodified legumin only 4–7 residues became unburied after glycosylation, it is probable that the inner part of the protein kept its close packed structure.

The influence of glycosylation on the secondary structure was studied by circular dichroism (Figure 8). Analysis of the CD spectra of the unmodified legumin using the method of Brahms and Brahms (1980) led to 9% α-helix, 46% β-sheet, and 45% random coil structures (Figure 8b). Compared to the method of Chen et al. (1972, 1974) (Figure 8a), it led to a more accurate fitting. Our results are in good agreement with those of Zirwer et al. (1985) obtained in the case of many 11S-type plant globulins, using the fitting method of Provencher and Glöckner (1981).

In the case of glycosylated samples, the best fitting was obtained by the method of Chen et al. (1972, 1974) (Figure 8c,d). Glycosylation significantly increases the percentage of helical structure and decreases the β-sheet structure as compared to the unmodified molecule. The spectra exhibited a similar profile for all the glycosylated derivatives. The same observation was made for the UV spectroscopy studies. It might indicate that the secondary and tertiary structures were slightly modified even for low glycosylation degrees. This effect was not related to the glycosylation level.

CONCLUSION

This first study of glycosylation on seed storage proteins shows that 11S-type globulins can be highly modified by reductive alkylation due to their important content in lysyl residues. Up to 120 galactose residues can be covalently attached to one legumin molecule.

Whatever the nature of carbohydrate and the extent of modification, the structure of legumin is not much changed. As shown by ultracentrifugation, ultraviolet difference spectra, and circular dichroism, glycosylation seems only to induce a slight opening of the quaternary and tertiary structures. In addition, glycosylation tends to decrease the aggregated forms observed by electrophoresis for unmodified legumin and leads to the formation of partially dissociated molecules.

The kinetic studies of modification indicate that all the amino groups do not react at the same rate. Considering the complex structure of legumin, some amino groups are probably located at the surface of the protein, others being more buried. This assumption is reinforced by the fact that the legumin incorporates monosaccharides such as galactose and glucose both faster and more extensively than more voluminous molecules such as lactose. For the same reasons of accessibility of the reagent, the TNBS method only measured about 50% of amino groups present in the unmodified legumin molecules.

The high degrees of glycosylation obtained under our experimental conditions completely modify the hydrophilic/hydrophobic balance of the protein. Thereby, important changes can be expected in the functional properties which are now under investigation.

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