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Transglutaminase-Catalyzed Glycosylation of Vegetable Proteins. Effect on Solubility of Pea Legumin and Wheat Gliadins

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Transglutaminase was used to covalently attach glycosyl units to glutamine residues of legumin and β-gliadins. To prevent ε-(γ-glutamyl)lysine cross-link formation, the lysine residues of legumin and β-gliadins were first blocked by reductive alkylation. In this way, the percentage of modification of amino groups was 84% for legumin and 100% for β-gliadins. Then, transglutaminase action resulted in incorporation of 18 and 57 glycosyl units per mole of alkylated β-gliadins and legumin, respectively. The corresponding degree of glycosylation of glutamine residues was 15.7% with gliadins and 25.7% with legumin. The solubility of neoglycoproteins was markedly increased over that of native proteins in the range of their isoelectric points (pH_i). This effect was much less pronounced for pH_s far from the pH_i. For pH values below 5.0, the solubility of glycosylated β-gliadins was even slightly lower than that of native β-gliadins.

INTRODUCTION

Glycosylation, one potential means of improving the functional properties of proteins (Canton and Mulvihill, 1983; Kitabatake et al., 1985; Courthaudon et al., 1989), appears to be of great interest for vegetable proteins presenting low solubility. Thus, we have recently shown that neoglycoproteins, prepared from pea legumin, were largely more soluble and exhibited a higher viscosity and better surface properties than native protein (Caer et al., 1990a; Baniel et al., 1992). Unfortunately, the glycosylated derivatives of proteins are usually synthesized by chemical methods [for a general review, see Stowell and Lee (1980)]. These are of limited application in food technology because it is difficult to control the extent of glycosylation, the sites of attachment of carbohydrate, and the secondary reactions and also to completely eliminate the toxic reagents eventually used in the chemical modification of proteins.

The enzymatic methods are capable of overcoming all of these difficulties. Three types of enzymes can be considered to catalyze the reaction of glycosylation: glycosyltransferases, glycosidases, and transglutaminases. Glycosyltransferases (EC 2.4) are responsible for *in vivo* glycosylation reactions, but so far their *in vitro* utilization is hampered by the lack of commercially available enzymes capable of directly catalyzing the attachment of carbohydrates or oligosaccharides to side chains of amino acid residues. Moreover, the necessity of cofactors and the very high specificity of glycosyltransferases toward the glycosyl donor and acceptor would not allow the use of these enzymes as a general method of *in vitro* glycosylation of food proteins. Glycosidases (EC 3.2) do not present these inconveniences. They are easy to purify, commercially available, and inexpensive. In addition, glycosidases generally possess a good thermal stability and display very low specificity toward glycon acceptor. For these reasons, more and more works are devoted to their transglycosylation and synthesis properties. However, at the present time, only the syntheses of oligosaccharides, various glycosidic derivatives [for review, see Nilsson (1991)] and glycosyl-L-serine (Cantacuzene et al., 1991; Sauerbrei and Thiem, 1992; Holla et al., 1992) have been successfully catalyzed by these enzymes. As for transglutaminases (EC 2.3.2.13), they catalyze a Ca^{2+}-dependent acyl-transfer reaction between the γ-carboxyamide groups of peptide-bound glutamine residues and a variety of primary amines

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(Folk and Chung, 1973; Folk and Finlayson, 1977). This reaction leads to the posttranslational modification of proteins through either the formation of intra- and intermolecular isopeptide bonds via ϵ-(γ-glutamyl)lysine bridges or the covalent attachment of amines such as polyamine or putrescine (Gross et al., 1977; Lorand et al., 1979; Folk, 1980; Russel and Womble, 1982; Cariello et al., 1990). *In vitro*, transglutaminase can recognize a wide variety of alkylamines (Yan, 1987). This property has been largely exploited for fortifying food proteins by incorporation of limiting essential amino acids (Ikura et al., 1981, 1984, 1985; Bercovici et al., 1987). Taking advantage of this nonspecificity for donor, Yan and Wold (1984) have shown that oligosaccharide extended chemically with an alkylamine could serve as an amine donor for transglutaminase. In this way, glycosyl units have been covalently linked to glutamine residues in β-casein after blocking of lysine residues by amidination or acetylation to prevent cross-link formation (Yan and Wold, 1984). These works were performed to comprehend the role of the spacing of the carbohydrate moieties on the protein backbone in lectin–sugar interactions.

In the present study, guinea pig liver transglutaminase was used for glycosylating vegetable proteins with the view of improving their solubility. Legumin and gliadins were chosen because of their interesting role as functional agents as well as for their very different structural features. Legumin, the 11-S storage protein of pea (*Pisum sativum*) seeds, is a globulin with a compact oligomeric structure characterized by an arrangment of six (α,β) subunits (Plietz et al., 1983) and a relative molecular weight (M_r) of about 360 000 (Derbyshire et al., 1976; Casey, 1979). Gliadins of common bread wheat (*Triticum aestivum*) are complex mixtures of proteins which can be separated in different classes (α,β,γ,ω) on the basis of their electrophoretic mobilities (Bushuk and Zillman, 1978; Kahn et al., 1983). These proteins, very rich in proline, present rather nonordered structures. They are also characterized by their high content in glutamine residues, which represent about 40% of total amino acid residues (Popineau, 1985). Thus, gliadins appear to be substrates well adapted to their modification by transglutaminase. It should be noted that a possible role of this enzyme, present in the small intestine, has been suggested in the pathogenesis of coeliac diseases in cross-linking gliadins to cell and subcellular membranes (Bruce et al., 1985).

MATERIALS AND METHODS

Materials and Chemicals. Legumin was isolated from pea seeds (*P. sativum* L.) and purified as described by Gueguen et al. (1984) and Larre and Gueguen (1986). Crude gliadins were isolated from common wheat flour (*T. aestivum*), and the β-fraction was purified as described by Popineau and Pineau (1985). Guinea pig liver transglutaminase (2–4 units/mg of protein), 6-aminohexyl 1-thio-β-D-galactopyranoside, and dithiothreitol were obtained from Sigma Chemical Co. Sodium cyanoborohydride and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Aldrich Chemical Co. *N*-Carbobenzoxy-L-glutaminyl-L-glycine was from Bachem Fine Chemicals, and carbohydrates were from Merck. All other reagents and chemicals were of analytical grade.

Blocking of Amino Groups. Protein amino groups were blocked by reductive alkylation according to a modification of the procedure of Gray (1974). The reaction was carried out at 37 °C for 65 h in 45 mL of 0.12 M sodium bicarbonate (pH 8.5) containing sodium cyanoborohydride (26.4 mmol), galactose (8.6 mmol), and either legumin (1 μmol) or β-gliadin fraction (12.9 μmol). In the latter case, the medium also contained 10% dioxane. After the reaction, legumin solutions were dialyzed at 4 °C against 0.12 M sodium bicarbonate for 12 h and then against 0.12 M ammonium bicarbonate for 36 h with four changes of the buffer. Gliadin solutions were dialyzed against 0.12 M sodium bicarbonate containing 10% dioxane for 12 h and then against 0.01 M acetic acid (4 × 9 h). Finally, the solutions were freeze-dried.

Glycosylation of Legumin and Gliadin by Transglutaminase Reaction. Transglutaminase-catalyzed attachment of glycosyl units into legumin and gliadins was carried out at 37 °C in 20 mL (final volume) of 0.1 M Tris-HCl buffer (pH 8.1) containing legumin (0.278 μmol) or β-gliadin fraction (2.85 μmol), $CaCl_2$ (100 μmol), dithiothreitol (100 μmol), and 6-aminohexyl 1-thio-β-D-galactopyranoside (1 mmol). In the case of gliadins, the reaction mixture also contained 10% dioxane. Reaction was initiated by transglutaminase: two aliquots of 0.6 unit were added at 2-h intervals, followed by a third addition of 0.8 unit after a new 2-h interval. Then, the reaction was continued overnight. Finally, the solutions were extensively dialyzed at 4 °C against either 0.2 M NaCl (legumin) or distilled water (gliadins).

Control assays were also performed either without transglutaminase or with monodansylcadaverine, a well-characterized amine donor substrate (Lorand et al., 1968).

One enzymatic unit was defined as the amount of protein that catalyzes the formation of 1 μmol of hydroxamate/min from *N*-carbobenzoxy-L-glutaminyl-L-glycine and hydroxylamine at pH 6.0 and 37 °C.

Analytical Methods. The ketoamine linkages formed by reductive alkylation are acid-stable. Therefore, the amount of galactose coupled to proteins in this way was measured according to the method of Hantzsch (Nash, 1953) as modified by Gallop et al. (1981). The amount of carbohydrate attached to proteins by transglutaminase reaction was determined by the phenol–sulfuric acid method (Dubois et al., 1956). Protein concentrations were evaluated by the method of Lowry et al. (1951) using unmodified legumin and β-gliadin fraction as standards.

Assessment of Legumin and Gliadin Solubility. The solubility of the unmodified and glycosylated protein samples was determined according the method of Coffmann and Garcia (1977) modified as follows: solutions (4 mL) of legumin in 0.2 M NaCl and gliadins in distilled water were adjusted to pH values between 2 and 11 by adding 0.1 N HCl or 0.1 N NaOH; then the reaction volumes were brought to 5 mL. After 1 h of continuous stirring at room temperature, the pH of the solutions was again measured. This last value was reported as the solution pH. Protein concentration in the supernatants, obtained after centrifugation of solutions at 5000*g* for 30 min, was expressed as a percentage of the total protein concentration determined before centrifugation.

RESULTS AND DISCUSSION

Glycosylation of Native and Alkylated Legumin and Gliadin by Transglutaminase. Transglutaminase shows high affinity for straight-chain aliphatic amines of six carbons (Lorand et al., 1979). For this reason, 6-aminohexyl 1-thio-β-D-galactopyranoside, commercially available, was chosen as glycosyl residue. As observed in preliminary experiments, its incorporation into gliadins was always equal or superior to that of dansylcadaverine or maltosylcadaverine (Fournier et al., 1989). On the other hand, legumin and gliadins, naturally nonglycosylated, appear to be good candidates as acceptor proteins because of their large number of glutamine residues (potential sites of glycosylation by transglutaminase) (Table I).

When the native proteins were used, 4.5% and 6.1% of the glutamine residues were glycosylated into legumin and β-gliadin fraction, respectively (Table I). In this case, intermolecular cross-linking via ϵ-(γ-glutamyl)lysine bridges occurred at the same time and led to the formation of large molecules which were unable to penetrate into a polyacrylamide gel during electrophoresis.

To prevent such cross-links, the protein amino groups were blocked by reductive alkylation. Under our experimental conditions, 84% of legumin amino groups were modified (Table I). The degree of blocking of amino groups

Table I. Incorporation of Galactosyl Units into Legumin and β-Gliadin Fraction by Transglutaminase

protein	total no. of amino groups,[a] mol/mol of protein	deg of alkylation of amino groups, %	total no. of glutamine residues, mol/mol of protein	galactosyl units incorpd, mol/mol of protein	deg of modification of glutamine residues, %
unmodified legumin	144	0	222	10	4.5
alkylated legumin	144	84	222	57	25.7
unmodified β-gliadin fraction	3	0	115	7	6.1
alkylated β-gliadin fraction	3	100	115	18	15.7

[a] Based on the sum of ε-amino (lysyl) and α-amino groups.

of this protein obtained by citraconylation (Larre et al., 1992) was very similar (86%). It should be noted that the structure of legumin is little changed by reductive alkylation (Caer et al., 1990b), whereas citraconylation leads to a stepwise dissociation of the 12.5-S sedimenting native legumin into 7.4- and 3.3-S components (Larre et al., 1992). Thus, a greater accessibility of glutaminyl residues related to conformational changes could explain the higher degree of transglutaminase-catalyzed incorporation of putrescine (89 mol/mol) after citraconylation than of glycosyl residues (57 mol/mol) after alkylation of legumin.

As deduced from values given in Table I, the degree of modification by transglutaminase after blocking of amino groups was multiplied by factors 5.7 for legumin and only 2.6 for the β-gliadin fraction. The lowest increase observed with the β-gliadin fraction can be explained by the limited number of free amino groups in the native molecules which are, for this reason, less susceptible than legumin molecules to form cross-links with transglutaminase. Nevertheless, the intrinsic degree of modification of the glutamine residues in the β-gliadin fraction (native or alkylated) appears to be relatively low for molecules without globular structure. In fact, it is known that transglutaminase is specific for only a limited number of glutamine residues in the acceptor protein (Brenner and Wold, 1978; Folk, 1983). Gorman and Folk (1980, 1981) showed that determinants for enzyme recognition are contained in the primary structure surrounding the substrate glutamine residues and that certain amino acid residues are more important than others in directing the action of transglutaminase. Thus, from 21 glutamine residues in β-casein, only 5 are primary acceptors and 2 secondary acceptors (Yan and Wold, 1984). Five of these seven residues have hydroxy amino acids as adjacent amino acids, namely serine, threonine, or tyrosine. However, other glutamine residues, reactive in different proteins, have quite different neighboring residues (Tamaki and Aoki, 1982; Berbers et al., 1983, 1984; Bowness et al., 1987; Simon and Green, 1988; Coussons et al., 1992). In addition, the reactivity of any glutamine residues may be influenced not only by its immediate neighbors but also by remote amino acid residues that could alter protein folding (Simon and Green, 1988). These authors showed that of 150 glutamine residues in intact human involucrin, one is highly preferred by the keratinocyte transglutaminase as the amine acceptor, but many peptides, prepared from a tryptic digest of the same protein, are able to act as amine acceptor (Etoh et al., 1986).

Solubility of Native and Glycosylated Legumin and Gliadin. The native conformation of legumin is known to be dependent on ionic strength and pH. Ionic strength has to be kept above 0.1 M to prevent aggregation. On the other hand, extreme pH conditions, especially at acidic pH, induce the irreversible dissociation of pea legumin (Gueguen et al., 1988). The solubility patterns of native and glycosylated legumin at different pH values and at constant ionic strength (0.2 M NaCl) are shown in Figure 1. Glycosylated legumin exhibited a solubility better than that of native legumin in the pH range between 3.5 and

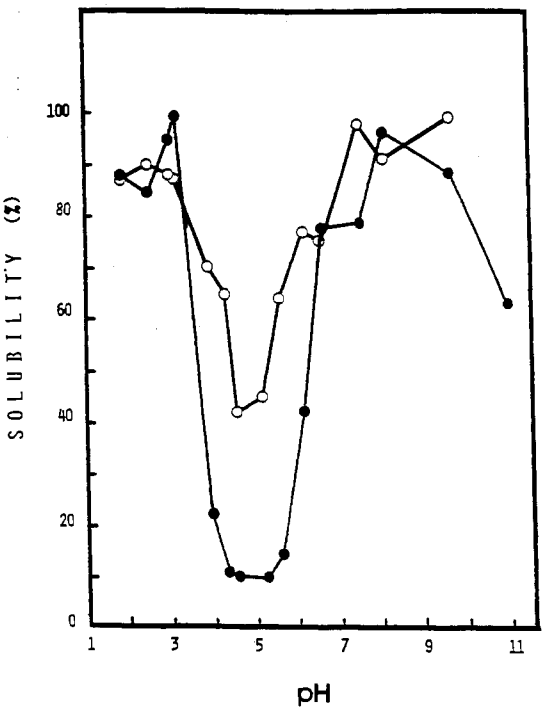

Figure 1. Influence of pH on the solubility of native (●) and galactosylated (○) legumin.

8.0. The increase of solubility is particularly marked in the pH_i region. For example, at pH 5.5, the solubility was 65% for glycosylated legumin compared to 12% for native protein. In addition, we have previously shown that the modification of legumin by blocking only the lysyl residues induced a shift of the pH_i to more acidic values (Caer et al., 1990a). After transglutaminase action, no effect of this type was observed.

The solubility curve, shown in Figure 2, presents a V profile much less marked for glycosylated protein than for the native β-gliadin fraction. Thus, at pH_i (pH 6.3), the solubility increased from 28% to 60% by glycosylation of gliadins. In addition, the solubility of glycosylated derivatives remained between 60% and 85% in the pH range between 2.0 and 10.5.

Conclusion. The reaction catalyzed by transglutaminase offers an efficient method for selective incorporation of functional groups into proteins under mild conditions. In this work, this property was utilized to bind glycosyl units to vegetable proteins, namely gliadins and legumin. In this way, 18 and 57 mol of glycosyl units were incorporated/mol of β-gliadin fraction and legumin, respectively. When compared to native proteins, the neoglycoproteins have a better solubility mainly round pH_i. The method is especially interesting for gliadins which possess very little of free amino groups. In the case of legumin, blocking of these groups is necessary to avoid cross-linking reactions. A study is now in progress to block these groups by a reversible treatment without damage to the conformation of legumin. Then it will become possible

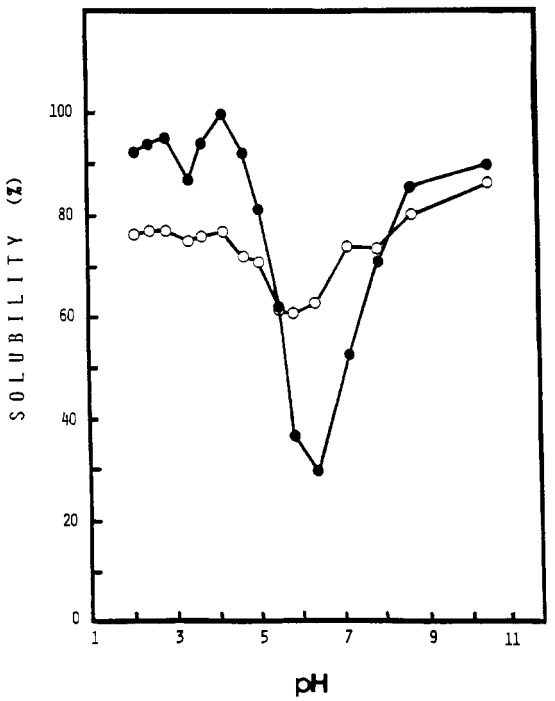

Figure 2. Influence of pH on the solubility of native (●) and galactosylated (○) β-gliadin fraction.

to study the single influence of the transglutaminase-catalyzed modification on the solubility of legumin.

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