Protease Susceptibility and Amino Group Accessibility to Trinitrobenzenesulfonic Acid of Legumin during Its Glycosylation

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In vitro digestibility by trypsin and α-chymotrypsin of neoglycolegumins, prepared by reductive alkylation of amino groups of pea legumin, was measured for various degrees of glycosylation. With a low extent of glycosylation, the initial rates of hydrolysis by α-chymotrypsin were dramatically increased compared to native legumin. When trypsin was used, no increase of the initial rates occurred, because the hydrolysis of new specific cleavage sites, which may have been exposed by a slight expansion of the protein structure, was counterbalanced by a great decrease of rates of hydrolysis of the glycosylated-lysyl peptide bonds. For high glycosylation degrees, the rates of hydrolysis decreased compared to native legumin with both trypsin and α-chymotrypsin. Steric hindrance increased as the size of carbohydrate and the degree of glycosylation increased, which predominated over structure expansion in limiting hydrolysis. In native legumin, about 50% of amino groups are accessible to trinitrobenzenesulfonic acid. A progressive decrease of the inaccessible amino groups occurred during glycosylation. Since the inner part of the neoglycoprotein keeps its close packed structure, our results suggest that amino groups are located in the relatively outer part of the molecule.

INTRODUCTION

Legumin, one of the major storage proteins of pea, has a complex globular structure composed of six (α,β) 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). Like most seed proteins of 11S type, its utilization in the food industry is limited, primarily because of its low solubility. In a previous study, we showed that in vitro glycosylation of legumin completely modified the hydrophilic/hydrophobic balance of the molecule with consequent improvement in its solubility and emulsifying properties (Caer, 1991; Baniel et al., 1992).

By reductive alkylation, 84% of amino groups of legumin may be progressively galactosylated, although about 50% of these groups are inaccessible to 2,4,6-trinitrobenzenesulfonic acid (TNBS) in the native molecule (Caer et al., 1990a). Considering the complex structure of legumin, these results suggest that glycosylation leads to a significant modification of the protein structure and unmasks lysyl residues probably buried and not very accessible to TNBS in the native molecule.

However, data obtained by ultracentrifugation and analyses of ultraviolet difference spectra show that glycosylation of legumin seems to induce only a slight opening of the quaternary and tertiary structures. This effect was independent of the nature of carbohydrate (mono- or disaccharide) and the extent of glycosylation (Caer et al., 1990b). The ultraviolet difference spectra indicated that, among the 35 inaccessible tyrosyl residues in the native legumin, 4–7 residues became accessible after glycosylation.

Another approach to elucidate if the protein structure is compact or expanded is to study the in vitro digestibility of the protein by proteases. Waniska and Kinsella (1984) showed that a maltosylated derivative of β-lactoglobulin, prepared by chemical modification of amino groups, was hydrolyzed more quickly by trypsin than native β-lactoglobulin, although the number of sites available for trypsin decreased after modification. This result was explained by the fact that glycosylation of β-lactoglobulin unmasked basic amino acid residues either from the central region of the molecule or from dissociation of its quaternary structure. On the contrary, the in vitro digestibility of chemically modified caseins by covalent attachment of amino acids (Puigserver et al., 1979; Gaertner and Puigserver, 1984), reducing sugars (Lee et al., 1979), or aldehydes and ketones (Sen et al., 1981) to ε-amino(lysyl) residues was, in all cases, lower than that of unmodified protein. The initial rates of α-chymotrypsin-catalyzed hydrolysis of modified caseins decreased as the size of the modifying groups increased and as the degree of modification increased. Since casein has a much less compact structure than β-lactoglobulin, the decrease in rates of hydrolysis of casein derivatives was probably due to steric hindrance rather than significant conformational changes.

In this study, we looked at a structural approach to pea legumin by studying protease susceptibility and amino group accessibility by trinitrobenzenesulfonic acid of the protein during its glycosylation.

MATERIALS AND METHODS

Materials and Chemicals. Legumin was purified from pea flour (*Pisum sativum* var. Amino) as described by Gueguen et al. (1984) and Larre and Gueguen (1986).

Trypsin from bovine pancreas (type III, 10 500 BAEE units/mg of protein) was purchased from Sigma, and α-chymotrypsin from bovine pancreas (90 ATEE units/mg of protein) was obtained from Boehringer. Sodium cyanoborohydride and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were from Aldrich Chemical Co. All other reagents and chemicals were of analytical grade.

Glycosylation of Legumin. Carbohydrates (galactose, glucose, and lactose) were bound to legumin by reductive alkylation of amino groups using a modification of the procedure of Gray (1974). The reaction was carried out at 37 °C for 65 h in 9 mL of 0.12 M sodium carbonate (pH 8.5) containing legumin (0.2 μmol), sodium cyanoborohydride (5.28 mmol), and carbohydrate (1.86 mmol). The solutions were then dialyzed at 4 °C against 0.12 M sodium bicarbonate for 48 h with five changes of the buffer.

In addition, molar ratios of galactose/amino groups were varied between 0 and 60 to obtain various degrees of glycosylation.

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Table I. Effect of Nature of Carbohydrate on Initial Rates of Hydrolysis of Glycosylated Derivatives of Legumin by Trypsin and α-Chymotrypsin

substrate	carbohydrate bound, mol/mol of legumin	modified amino groups,[a] %	relative initial rates	
			trypsin	α-chymotrypsin
control legumin	0	0	100	100
galactosylated derivative	116	81	48	50
glucosylated derivative	80	56	61	69
lactosylated derivative	73	51	0	25

[a] Calculation based on the sum of ε-amino(lysyl) and α-amino groups (i.e., 144 total amino groups/mol of legumin).

Analytical Methods. The amounts of carbohydrates attached to legumin by reduced ketoamine linkage were determined according to the method of Hantzsch (Nash, 1953) as modified by Gallop et al. (1981). Protein concentrations were measured according to the method of Lowry et al. (1951) using unmodified legumin as standard.

Amino groups were quantified either by the method of Fields (1972) using TNBS under nondenaturing conditions or by the method of Moore and Stein (1954) using ninhydrin under denaturing conditions.

Enzymatic Methods. The initial rates of hydrolysis of legumin and legumin derivatives were determined by measuring the amount of free amino groups using the ninhydrin method (Moore and Stein, 1954). The reaction mixtures containing 0.1% (w/v) native legumin or glycosylated legumin and 2 mM calcium chloride in 0.12 M sodium carbonate (pH 8.5) were incubated at 37 °C with 1/500 (w/w) trypsin or 1/100 (w/w) α-chymotrypsin.

Aliquots (100 μL), removed at 30-s intervals for a period of 5 min, were immediately mixed with 100 μL of 1 M sodium acetate buffer (pH 5.5) cooled in ice.

RESULTS AND DISCUSSION

Initial Rates of Hydrolysis by Trypsin and α-Chymotrypsin. Table I shows that the initial rates of α-chymotrypsin-catalyzed hydrolysis were always lower with highly glycosylated derivatives of legumin than with native protein. Besides, the size of carbohydrate seems to directly govern this decrease. Thus, although the degree of glycosylation of lactosylated derivative was lower, it was more slowly hydrolyzed by α-chymotrypsin than galactosylated and glucosylated derivatives. This decrease in digestibility is probably due to steric hindrance, produced by the carbohydrate residues bound to the protein, which prevents the approach of the protease.

It should be noted that trypsin gave results similar to those of chymotrypsin (except with lactosylated derivative), although the modification of lysyl residues leads to a large decrease of tryptic digestion sites (Lee et al., 1979; Waniska and Kinsella, 1984). Therefore, glycosylation might induce a better accessibility of hydrophilic or charged groups such as lysyl and arginyl residues. However, the conformational changes would be insufficient to expose hydrophobic groups, such as aromatic residues, which would be buried in the core of the protein. To test this hypothesis, the effect of the degree of glycosylation on the initial rates of hydrolysis of galactosylated derivatives was investigated using trypsin and α-chymotrypsin as proteases.

As shown in Table II, with a low extent of glycosylation, the initial rates of hydrolysis by α-chymotrypsin dramatically increased, compared to those of unmodified legumin. These results suggest a slight expansion of the tertiary structure or a partial dissociation of the quaternary structure, revealing a few additional sites of hydrolysis of the tyrosyl, tryptophanyl, and phenylalanyl peptide bonds. This interpretation is in agreement with ultraviolet difference spectra which indicate the presence of about 43 tyrosyl residues exposed to the surface of unmodified legumin and about 47 tyrosyl residues accessible after glycosylation (Caer et al., 1990b). When trypsin was used, no increase of the initial rates occurred because the access to specific sites blocked by glycosylation of lysyl residues counterbalanced the better accessibility of other specific sites.

Table II. Effect of Degree of Glycosylation on Initial Rates of Hydrolysis of Galactosylated Derivatives of Legumin by Trypsin and α-Chymotrypsin

substrate	galactose bound, mol/mol of legumin	modified amino groups,[a] %	relative initial rates	
			trypsin	α-chymotrypsin
control legumin	0	0	100	100
galactosylated derivatives	28	19	80	142
	49	34	90	142
	88	61	80	107
	116	81	48	50
	122	85	44	48

[a] Calculation based on the sum of ε-amino(lysyl) and α-amino groups (i.e., 144 total amino groups/mol of legumin).

For high glycosylation degrees, the digestibility decreased with trypsin and α-chymotrypsin as well (Table II). Steric hindrance induced by the galactose residues bound to the protein surface predominated over molecule expansion. Finally, in vitro digestibility tests confirm the very slight opening of the quaternary and tertiary structures previously deduced by ultracentrifugation and ultraviolet spectroscopy (Caer et al., 1990b). Our overall results indicate that the inner part of the protein keeps its close packed structure, which appears to be relatively rigid, and consequently does not undergo notable changes, even after a high degree of glycosylation. On the other hand, the initial rates of tryptic hydrolysis of maltosylated derivatives of β-lactoglobulin were always higher than those observed with native protein (Waniska and Kinsella, 1984). Thus, although β-lactoglobulin is a globular protein, its structure is probably less rigid and compact than that of legumin and allows extensive conformational changes during glycosylation.

Accessibility of Unmodified Amino Groups to TNBS. Estimation of the number of amino groups accessible to TNBS can give complementary information about the structural changes of legumin during glycosylation. As notified by Sen et al. (1981), proteins having a tertiary structure possess amino groups more or less buried which cannot be detected by the TNBS method described by Fields (1972). Considering the sum of ε-amino (lysyl) and α-amino groups, 144 amino groups can be determined in one molecule of legumin on the basis of its $(\alpha,\beta)_6$ structure and using the primary sequence proposed by Lycett et al. (1984). However, in the native molecule, only 64 amino groups were accessible to TNBS. As shown in Figure 1, a progressive decrease of the inaccessible amino groups occurred during glycosylation. In addition, under optimal experimental conditions, 122 amino groups can be galactosylated (Caer et al., 1990b), suggesting important structural changes, contrary to conclusions obtained from UV difference spectra and digestibility studies. Such a discrepancy can be justified if amino groups are only superficially buried while aromatic residues are mainly located in the central region of the molecule.

Conclusion. The study of legumin proteolysis and of amino group accessibility during glycosylation would indicate that the inner part of this 11S type globulin has a rigid structure. Glycosylation only leads to a peripheral opening of the protein molecule. Protease attack is not threatened by low glycosylation degrees because the

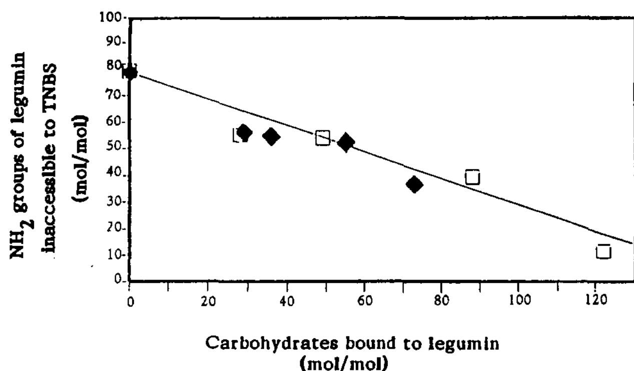

Figure 1. Effect of degree of glycosylation on the number of amino groups inaccessible to TNBS in galactosylated (□) or lactosylated (◆) derivatives of legumin.

superficial expansion of the structure improves the access to most of the amino groups and of some aromatic residues. Thus, under conditions of limited glycosylation, the initial rate of hydrolysis catalyzed by α-chymotrypsin is increased. The same phenomenon cannot be observed with trypsin because the hydrolysis of new specific cleavage sites is counteracted by a large decrease of the rate of hydrolysis of the glycosylated-lysyl peptide bonds. In all cases, high degrees of glycosylation cause a steric hindrance which makes enzyme approach very difficult and even impossible when disaccharides are bound to the legumin molecule. Finally, these results are in good agreement with those previously obtained by spectral studies (Caer et al., 1990b) which suggested that the structure of legumin is little changed by glycosylation.

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