Effect of Bound Carbohydrate on the Action of Trypsin on Lupin Seed Glycoproteins

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Proteolysis by trypsin of the storage proteins of lupin seed is less extensive than that of bovine serum albumin or of casein, and the decrease is related to the amount of carbohydrate bound to the protein. Purified conglutin γ and vicilins 4 and 6, the three seed globulins having the highest sugar content, were first incubated with jack bean exoglycosidases, which removed 75, 81, and 80% of the carbohydrate, respectively. This increased the subsequent action of trypsin in liberating small trichloroacetic acid (TCA) soluble peptides and/or in forming large TCA-insoluble fragments evidenced only by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The increase was related to the amount of sugar detached. The effects observed may depend on the removal of a steric hindrance due to the bound carbohydrate and on other additional mechanisms that involve the relationship between the amount of bound sugar and extent of hydrophobic areas on the protein surface.

Various findings reported in the literature indicate that seed proteins are less attacked by mammalian digestive endopeptidases and are frequently less digestible than animal proteins (Kakade, 1974; Lynch et al., 1977a,b; Romero and Ryan, 1978; Schwimmer, 1975). Factors responsible at a molecular level for this behavior include primary structure, amino acid content, and protein conformation (Boonvisut and Whitaker, 1976; Fukushima, 1968; Kakade, 1974; Lynch et al., 1977a,b; Romero and Ryan, 1978; Schwimmer, 1975).

The findings mentioned refer to seed proteins in general. A number of these are glycoproteins, and reduced activity of endopeptidases has been described on glycoproteins (Anantha Samy, 1967; Christensen and Birkeland, 1974; Churchill and Hokin, 1976; Gottshalk and Fazakas de St. Groth, 1960), including artificially glycosylated enzymes (Christensen and Vergarud, 1974; Marsh et al., 1977). The nonglycosylated counterparts were degraded more easily, the difference being attributed to steric hindrance of the protease by the saccharide chain (Lamport, 1980; Marsh et al., 1977) or to a conformational rearrangement in the protein, induced by modification of the charge distribution when the charged residues of sialic acid were detached (Anantha Samy, 1967). Removal of sialic acid, however, does not always increase trypsin action (Churchill and Hokin, 1976).

Lupin seed globulins all contain bound carbohydrates (Duranti et al., 1981; Eaton-Mordas and Moore, 1979). In previous work we have shown that pepsin, and to a larger extent trypsin, is less effective on the globulins than on casein and on bovine serum albumin (BSA). The decrease in activity paralleled the sugar content in each separated globulin (Cerletti et al., 1983). Sialic acid is not contained in lupin globulins, their bound carbohydrate being mostly neutral sugar with some *N*-acetylglucosamine (Duranti et al., 1981; Eaton-Mordas and Moore, 1979). The amount of bound sugar is relatively small, the largest being, in the variety of lupin studied in this paper, 8.8% by weight in conglutin γ (globulin 1). It seemed interesting to establish whether the carbohydrate component was involved in the mentioned decrease of trypsin action. Conglutin γ, vicilin 4, and vicilin 6 were studied; these proteins have the highest bound sugar and are the most abundant globulins in the seed. Glycosidases were used to remove the bound sugar with the least possible modification of the protein. The different approaches used in the work and the results obtained are reported in this paper.

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MATERIALS AND METHODS

Chemicals were of the highest purity available commercially. Trypsin (EC 3.4.21.4), crystallized twice from bovine pancreas, type III, dialyzed, and lyophilized, was from Sigma Chemical Co.

Universal buffer (diethylbarbituric acid, citric acid, KH_2PO_4, and boric acid plus NaOH) was prepared as described by Long (1968). When used, it was 28.75 mM. The concentrations of all reagents mentioned refer to the total sample volume.

Lupinus albus of the sweet Multolupa variety was used. Seed globulins were extracted and purified as previously described (Duranti et al., 1981).

Total free amino acids were determined by the ninhydrin reaction (McCaldin, 1960); the standard from Sigma Chemical Co. was used. Microbiuret assays were done according to Itzaki and Gill (1964) by using BSA as the standard. Compounds separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie Blue (Dixon and Coherle, 1981) were scanned at 610 nm. Apparent molecular weights (M_r) were determined with reference to known standards. Neutral sugars were determined according to McKelvy and Lee (1969). Other materials and assays were as previously described (Duranti et al., 1981; Restani et al., 1981).

Glycosidases were extracted from jack bean flour according to Li and Li (1970) and purified to precipitation with ammonium sulfate. The precipitate, dissolved in 0.1 M phosphate buffer, pH 7.0, was desalted on Sephadex G-50, concentrated in an Amicon 202 ultrafiltration cell, and kept frozen in liquid nitrogen until used. As detailed under Results, α-mannosidase, β-galactosidase, and β-*N*-acetylglucosaminidase were present in this preparation, which also displayed very weak proteolytic activity. The activity of each glycosidase under saturating conditions, on a suitable *p*-nitrophenyl substrate from Koch Light, Ltd., 1 mM, was assayed in 50 mM citrate and in universal buffer at pH 4.5, 3.5, and 5.0, respectively (Li and Li, 1968, 1970). Incubation was at 25 °C for 5–10 min. Enzyme action was stopped as described by Li and Li (1970) and *p*-nitrophenol liberated was measured at 450 nm. Lupine proteins (3 mg/mL) were incubated in universal buffer pH 4.5, and conglutin γ was also incubated at pH 3.5.

Proteolytic activity was assayed at 37 °C on benzoyl-arginyl-*p*-nitroanilide (BAPA) from Merck and on a protein substrate. BAPA was 0.33 mM in 0.05 M sodium phosphate buffer, pH 7.0. The *p*-nitroanilide formed was monitored at 405 nm; E_M = 9620 was used (Wachsmuth et al., 1966). The protein substrate (3 mg/mL) was either casein or BSA or a lupin protein, the protein/enzyme ratio

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(w/w) being 60/1. It was digested in universal buffer, pH 7.9. Aliquots were withdrawn at intervals ranging from 15 min to 24 h and the reaction was stopped either by precipitating the protein in 10% trichloroacetic acid (TCA) or by diluting with the same volume of 0.5 M Tris-HCl buffer, pH 6.8, containing 15% glycerol and 2% SDS and then heating for 10 min at 100 °C. The latter samples were analyzed by SDS–PAGE. Those precipitated with TCA were centrifuged for 30 min at 13000g, the biuret or ninhydrin-reactive compounds then being determined on the supernatant.

The universal buffer does not interfere with microbiuret determination of proteins or with that of amino acid mixtures with ninhydrin. Trypsin activity on 0.33 mM BAPA was 15% less in universal buffer, pH 7.9, than in 0.1 M phosphate buffer at the same pH, with or without additions of 100 mM mannose or maltotriose. In a statistical treatment of trypsin hydrolysis data from various casein preparations, the variability coefficients were always less than 10% both for microbiuret and for ninhydrin assays, thus indicating the reliability of the procedures used.

RESULTS

Cleavage of Glycosidic Bonds. α-Mannosidase, β-galactosidase, and β-*N*-acetylglucosaminidase activities were in a ratio of 100:65:6.8 in the glycosidase preparation. The reaction developed linearly for 60 min, with or without added BSA or total extract of lupin globulins. Mannosidase activity increased by 4% in the universal buffer as compared to citrate while galactosidase and *N*-acetylglucosaminidase were unchanged.

In the Multolupa variety of *L. albus* the vicilins 4 and 6 contain respectively 4.0 and 4.9% bound carbohydrate (w/w), which is 95% mannose (Duranti et al., 1981): they were incubated in a ratio of 70:1 (w/w) with the glycosidase preparation. In this condition the enzymes were in great excess with respect to the amount of bound sugar, and 81.0 and 80.4% of sugar were liberated, respectively. For conglutin γ the ratio to glycosidase preparation was 35:1. This globulin contains 8.8% carbohydrate, which is mostly galactose (Duranti et al., 1981). Only 21.6% of the bound sugar was split at pH 4.5 whereas at pH 3.5, the optimal pH for galactosidase, 75% was detached.

After 60 min of glycosidase action, part of the sample was adjusted to pH 7.9 and incubated at 37 °C with or without trypsin. The rest was heated for 30 min at 100 °C, and the protein that sedimented in 20 min at 13000*g* was analyzed for residual bound sugar and protein content. This method allowed separation of protein from solubilized sugars. Protein recovered was about 90% of original, a higher value than that obtained after precipitation with TCA or dialysis.

Effect of Glycosidase Treatment on Proteolysis. The glycosidase preparation displayed weak proteolytic activity with the main characteristics of an exopeptidase (Figures 1B and 2B). Breakdown of BAPA and BSA at pH 7.9 and at pH 4.5, under saturating conditions, was 1.7% of that measured with trypsin. This activity did not interfere with determination of tryptic activity since proper blanks were made, as shown in the figures, and can be deducted. Lupin globulins, incubated without trypsin, with or without addition of glycosidases, appeared unmodified in peptide composition on SDS–PAGE (Figure 3). Protease inhibitors were not present in the glycosidase preparations nor they were associated with the seed proteins used as a substrate for trypsin.

As shown in the figures, trypsin alone had a limited effect on lupin proteins. Breakdown was particularly low

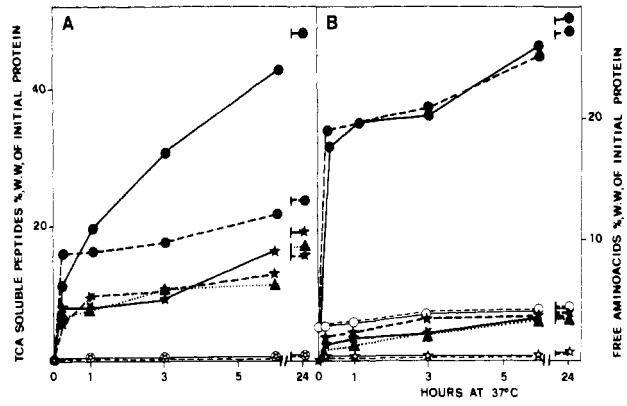

Figure 1. Release of TCA-soluble peptides (A) and free amino acids (B) from conglutin γ. The protein, 3 mg/mL, was incubated for 1 h at 25 °C, at pH 4.5 (dashed line) or pH 3.5 (solid line) with (circles) or without (stars) the glycosidase preparation (35:1 w/w). The pH was then adjusted to pH 7.9 (t = 0 in the figure), trypsin was added (1:60 w/w) to part of the preparation (full symbols), and the incubation with trypsin or without it (open symbols) continued at 37 °C. At the times indicated, aliquots were withdrawn and analyzed by the microbiuret (A) or the ninhydrin (B) reactions (see the text). Samples treated at 37 °C with trypsin without previous incubation at 25 °C are also shown (dotted line).

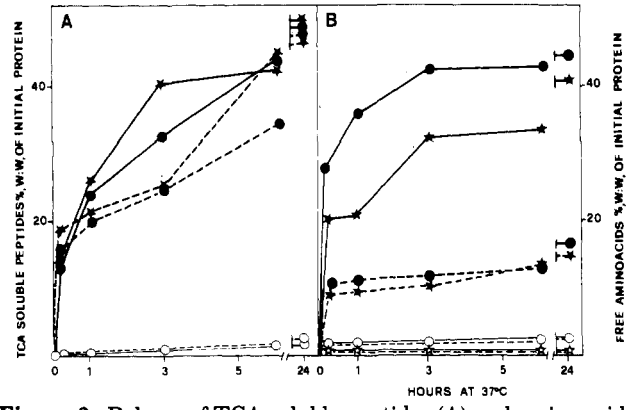

Figure 2. Release of TCA-soluble peptides (A) and amino acids (B) from globulins 4 and 6. Globulin 4 (solid line) and globulin 6 (dashed line) were incubated at pH 4.5 with or without glycosidases (70:1 w/w) and then at pH 7.9 with or without trypsin as described for conglutin γ in Figure 1. Other symbols are as in Figure 1.

for conglutin γ. Its action drastically increased after preincubation with glycosidases. For conglutin γ the effect was limited when glycosidases acted at pH 4.5, but it became significant after treatment at pH 3.5 (Figures 1A and 3). SDS–PAGE clearly shows that in the latter conditions the degradation of conglutin γ was already significant when trypsin had acted for only 15 min. After 6 h only some peptides of intermediate size, around 26 kilodaltons, were still present, while the major component was a peptide of 10 kilodaltons (Figure 3). This suggests that galactosidase, which has optimal activity at pH 3.5 (Li and Li, 1970), removed the most considerable hindrance to trypsin action.

The consistent and gradual increase of TCA-soluble peptides shown in figure 1A, when compared with the data of SDS-PAGE (Figure 3), indicates that, once the bound sugar was removed, fragmentation by trypsin took place gradually. Release of ninhydrin-reactive compounds was also increased but most of it developed in the early periods of incubation (Figure 1B), indicating that the exopeptidase action did not require an extended splitting of the original peptide.

In native globulins 4 and 6 the covalent continuity of the subunits is interrupted (Restani et al., 1981). The

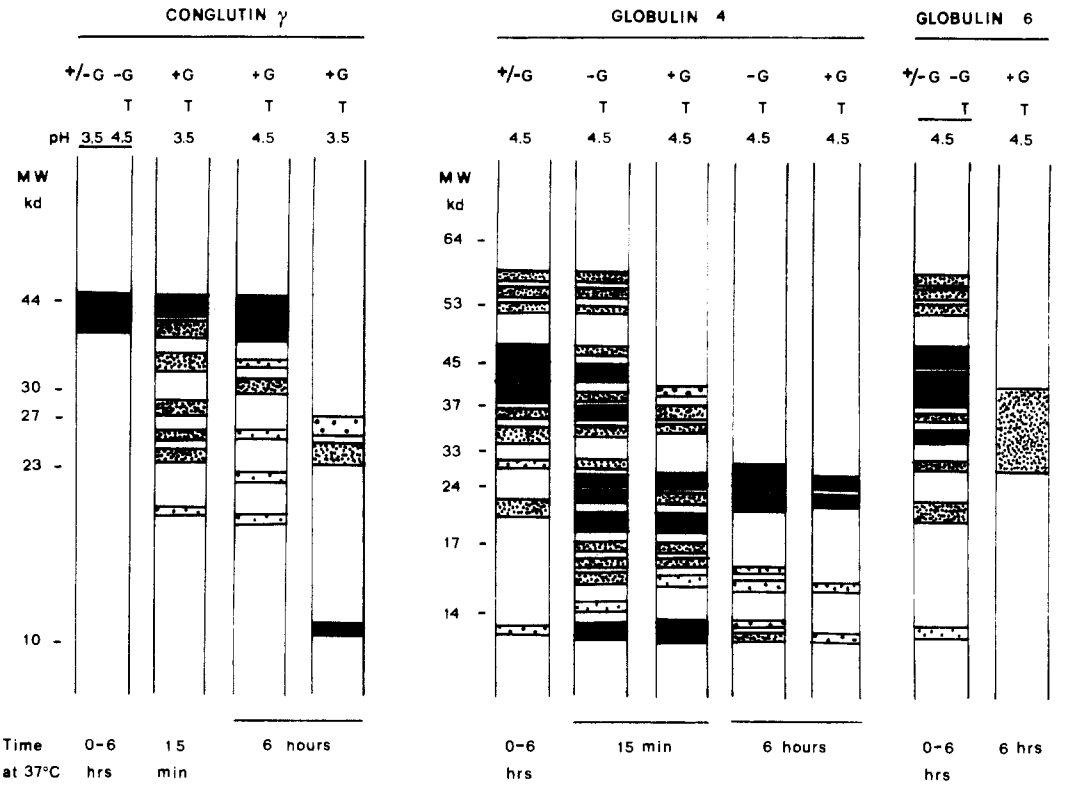

Figure 3. SDS–PAGE of conglutin γ and of globulins 4 and 6 submitted to various treatments. All samples were preincubated 1 h at 25 °C at pH 3.5 or 4.5 with (+G) or without (–G) glycosidase and then at 37 °C at pH 7.9 with (T) or without trypsin added. Only one sample is shown for conditions producing the same electrophoretic pattern.

peptide pattern did not appear to be modified by deglycosylation (Figure 3). Trypsin produced to a significant extent small TCA-soluble peptides from the native proteins. Treatment with glycosidases failed to increase this reaction significantly (Figure 2A). The large-sized peptides of globulin 4 were split by trypsin also without previous action of glycosidases, and the efficiency was considerably enhanced by deglycosylation (Figure 3). Those of untreated globulin 6 were not affected by trypsin but the protease acted on the deglycosylated protein (Figure 3). A specific effect of deglycosylation was thus apparent, which was not seen when only peptides soluble in TCA were measured. Deglycosylation also enhanced the production of free amino acids from globulin 4 but not from globulin 6 (Figure 2B).

DISCUSSION

Lupin does not contain antitryptic factors in the seed (Gallardo et al., 1974), and purified proteins were used in our experiments; the results here reported indicate that the protein-bound sugar is involved in reducing the action of trypsin. This explains previous data showing on the seed globulins of other lupin varieties a parallel between the carbohydrate content and decreased effect of trypsin (Cerletti et al., 1983).

The weak proteolytic activity of the glycosidase preparation does not represent a real problem: all data indicate that the glycosidases and not the protease(s) of the preparation were involved in increasing trypsin action.

Because of the specificity of the enzymes used and of the type of carbohydrate bound to lupin globulins (Duranti et al., 1981), neutral sugars were detached. Therefore, in these experiments the local change in conditions following removal of the charged sialic residue from animal glycoproteins, to which the modifications in protein conformation were in some cases attributed, is not likely to occur.

Studies with lipophilic probes indicate that lupin proteins have a significant number of hydrophobic residues on their surface, clustering into discrete hydrophobic areas. The amount decreases from conglutin γ to globulins 4 and 6 (Bonomi et al., 1983). The "hydrophobic" areas in the sugar residue, e.g., the glycosidic oxygen and the C–H groups in mannose, may interact with such hydrophobic peptide regions, giving them a hydrophilic "overcoat" (Lamport, 1980). Removal of this sugar coverage probably modifes the surface properties of the protein and its interaction with the medium and with the enzyme and possibly also its conformation. A role of bound carbohydrate in stabilizing the surface conformation has been shown for porcine ribonuclease (Wang and Hirs, 1977) and yeast external invertase (Chu et al., 1978). In this respect it is interesting that in the proteins studied here surface hydrophobicity parallels the amount of sugar and resistance to trypsin, and thus the effect of deglycosylation is more relevant where hydrophobic surface areas are more significant.

In *Lupinus angustifolius* the bound carbohydrate is supposed to consist of two chains of molecular weight about 5000 for conglutin β, which corresponds to our globulins 4 and 6, and of a limited number of chains of 1300 for conglutin γ (Eaton-Mordas and Moore, 1979). Steric hindrance due to the bound carbohydrate and the distribution of the oligosaccharide chains may play a role in directing the action of trypsin. It is interesting to note that in the native legumins and vicilins of *L. albus*, most of the carbohydrate is bound to large-sized protomers (Restani et al., 1981; Cerletti, 1983): if the endogenous endopeptidases are also influenced by the bound sugar, the presence of the carbohydrate may have impeded the posttranslational breakage of the original peptide close to the sites where the sugar is bound.

Once the sugar is removed, the action of trypsin increases on all proteins studied. Large fragments are formed. Formation of TCA-soluble peptides follows similar kinetics with all proteins considered, which is not the case before deglycosylation. TCA-soluble peptides are

small: molecular weights of about 330–380 were reported (Greenberg and Shipe, 1979). The determination of TCA-soluble peptides and amino acids alone therefore gives only a partial picture of the effects of the enzyme. Only SDS–PAGE made it possible to ascertain whether endopeptidase splitting with formation of large-sized fragments occurred.

Globulins 4 and 6 are similar in amino acid, sugar, and protomer composition, but their different molecular weights (Duranti et al., 1981; Restani et al., 1981; Cerletti, 1983) and surface hydrophobicity (Bonomi et al., 1983) suggest that the protomers are assembled differently. The different effect of trypsin indicates different availability of specific sites for the enzyme. The temperatures at which the tertiary structure of these proteins starts to break up indicate a looser conformation for globulin 4 (Bonomi et al., 1983). This may explain why the native protein is split by trypsin with formation of large and small peptides and of free amino acids. Also, the effect of deglycosylation is more pronounced, as indicated by the amino acids and insoluble peptides produced. Amino acid liberation from nondeglycosylated globulin 4 develops stepwise: this may indicate that, once acted upon by trypsin, the protein undergoes rearrangement before again being available to the enzyme. A similar situation has been described by Fukushima (1968) for the 11s globulin from the soybean, when attacked by the proteinase of *Aspergillus soyae*.

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Volatile Constituents of Carambola (*Averrhoa carambola* L.)

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Forty-one volatile components were identified from an extract of carambolas by using a capillary gas chromatograph-mass spectrometer combination. The most prominent aroma was grape-like, and methyl anthranilate was the most abundant component. The strong fruity aroma of the extract is probably due to the major esters and ketones in the extract.

Carambolas are consumed mostly as fresh fruit and have recently progressed from a dooryard ornamental to small commercial plantings of one or two selected cultivars (Brooks, 1983). The unique star-shaped fruit vary in color from white to dark yellow. The yellow varieties have more commercial appeal because the deeper yellow color is more attractive to the comsumer. The white varieties have been reported to be sweeter than the yellow varieties (Harler, 1983). Wagner et al. (1975) reported ascorbic and oxalic acid content, acidity, Brix, and taste panel evaluation for carambolas that were mostly of the yellow varieties. Flavor attributes ascribed to some of these were sweet, good, and apple-like, sour, tart, and apple-like, and sweet, good, and mild. Others have suggested carambolas have an apricot-like flavor (Harler, 1983). Little information on the chemical composition of carambolas is available, and to

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