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Binding of Condensed Tannins to Salivary Proline-Rich Glycoproteins: The Role of Carbohydrate

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Salivary proline-rich proteins have a high affinity for tannin and protect against the antinutritional effects of dietary tannins. Several of these proteins are glycosylated so we have investigated the role of the carbohydrate in their binding to tannin. The results suggest that oligosaccharides enhance the affinity and selectivity of binding to tannins and increase the solubility of the resulting tannin/glycoprotein complexes.

Protein/tannin interactions have been widely investigated with respect to the chemical nature of the interactions (Haslam, 1974; Oh et al., 1980). Among the features studied have been the stoichiometry of the complexes (Calderon et al., 1968), the effect of tannin chain length (Porter and Woodruffe, 1984), the ability of tannins to selectively bind certain proteins (Hagerman and Butler, 1981), and the effects of protein size, conformation, and amino acid composition (Hagerman and Butler, 1981). Carbohydrate/tannin interactions have also been examined (Davis and Hoseney, 1979; Deshpande and Salunkhe, 1982; McManus et al., 1985). However, interactions between glycoproteins and tannins have scarcely been studied. Strumeyer and Malin (1970) reported that yeast invertase (a glycoprotein) is resistant to inhibition by tannins and suggested that the carbohydrates of glycosylated enzymes may protect these glycoproteins against binding by tannins. Jones and Mangan (1977) reported that condensed tannin does not precipitate bovine submaxillary mucin at temperatures above 25 °C. They ascribed this lack of precipitation to the carbohydrate on the protein.

Tannins have been reported to be responsible for antinutritional effects including inhibition of digestive enzymes (Griffiths, 1979), formation of relatively less digestible complexes with dietary protein, depressed growth rates, and altered food consumption (Reddy et al., 1985). Rats and mice adapt to dietary tannin by the induced synthesis of several proline-rich (up to 44%) salivary proteins (PRPs) (Mehansho et al., 1983, 1985b). These proteins apparently diminish the antinutritional effects of dietary tannin by strongly binding to it. Hamsters do not produce salivary PRPs in response to dietary tannin and may be killed by tannin-containing diets to which rats and mice readily adapt (Mehansho et al., 1985a).

Many of these salivary PRPs contain carbohydrate in amounts up to 40% by weight (Mehansho and Carlson, 1983; Mehansho et al., 1985b). The strong affinity of these glycoproteins for tannins led us to examine the role of carbohydrate in the binding of tannins to these salivary proline-rich proteins.

MATERIALS AND METHODS

Bovine serum albumin (BSA), hen egg albumin, *Clostridium perfringens* neuraminidase, and SDS were obtained from Sigma (St. Louis, MO). Condensed tannin was purified from *Sorghum bicolor* L. Moench hybrid BR-64 (Hagerman and Butler, 1980) and from quebracho (Asquith and Butler, 1985). Crude quebracho powder was purchased from the Trask Chemical Co. (Marietta, GA)

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Table I. Relative Affinities of Native and Modified Rat Proline-Rich Protein GP_{158} for Sorghum and Quebracho Tannins

protein	relative affinity[a] sorghum	quebracho	protein	relative affinity[a] sorghum	quebracho
native GP_{158}	45	83.3	GP_{158} oligosaccharides	0	0
asialo GP_{158}	24.2	42.9	BSA	1	1
deglycosylated GP_{158}	11.2	11.2			

[a] Calculated by dividing the moles of [^{14}C]BSA in the assay by the number of moles of competitor needed to prevent 50% of the labeled protein from being precipitated (Asquith and Butler, 1985). Values are the average of duplicates.

and calf skin gelatin from Eastman Organics (Rochester, NY). Clostripain was obtained from Cappel Worthington, Bio-Gel A (1.5 m) was from Bio-Rad, and Sephadex G-50 and G-25 were from Pharmacia. Rat proline-rich glycoprotein GP-158 (Mehansho and Carlson, 1983) and mouse GP-66sm (Mehansho et al., 1985b) were purified from submaxillary glands as described elsewhere. B-9, a proanthocyanidin trimer, was a generous gift from Dr. E. Haslam, University of Sheffield, U.K.

Competitive Binding Assays. Conditions were adapted from Hagerman and Butler (1981). All reagent solutions were kept on ice until used. Varying amounts of competitor were mixed with 100 μg of [^{14}C]BSA (Jentoft and Dearborn, 1979) in 0.20 M acetate buffer (pH 4.8) for a total volume of 640 μL. To this were added 20 μg of tannin in 160 μL of methanol. This was sufficient tannin to precipitate 75% of the labeled protein in the absence of any competitor. The samples were mixed on a vortex mixer, incubated for 5 min at room temperature, and centrifuged (5 min, 800*g*). The resulting pellets were dissolved in two 100-μL volumes of 1% (s/v) SDS and counted in 4.0 mL of scintillation fluid.

Deglycosylation of Glycoproteins. Glycoproteins were deglycosylated by the procedure of Edge et al. (1981). To separate the oligosaccharides from the proteins, the deglycosylated proteins were chromatographed on a molecular sieve column (Bio-Rad A, 1.5 m) equilibrated with 50 mM ammonium bicarbonate. Peak protein fractions were combined and lyophilized. Previous studies have shown that deglycosylation did not alter the random-coil structure of these proteins (Mehansho, H., Kim, B., Westler, M., Carlson, D. M., unpublished observations, 1986).

Amino Acid Analysis. Protein and peptide concentrations were determined by amino acid analysis, as described previously (Muenzer et al., 1979).

Glycopeptide Preparation. Glycopeptides obtained by proteolysis with clostripain (Kauffman et al., 1982) were fractionated on Sephadex G-50 equilibrated with 50 mM NH_4HCO_3. Peaks were identified by absorbance at 230 nm and by the phenol–sulfuric acid reaction (Ashwell, 1966). Fractions containing glycopeptides and those containing nonglycosylated peptides were separately pooled and lyophilized.

Oligosaccharide Isolation. Mouse GP-66sm contains only O-linked oligosaccharides (Mehansho et al., 1985a) whereas rat GP-158 contains N-linked oligosaccharides (Mehansho and Carlson, 1983). O-linked oligosaccharides were released from mouse GP-66sm by alkaline sodium borohydride reductive elimination as described by Carlson (1968) and fractionated on Sephadex G-25 (equilibrated and eluted with 50 mM NH_4HCO_3). N-Linked oligosaccharides were prepared from rate GP-158 by the procedure of Zinn et al. (1978).

Asialo Glycoprotein Preparation. Sialic acid was removed from rate GP-158 by acid treatment. Glycoprotein (10 mg) was heated at 80 °C for 1 h in 0.1 N H_2SO_4. The sample was cooled and neutralized with NH_4HCO_3. The asialo glycoprotein was then desalted on Sephadex G-50 equilibrated with 50 mM NH_4HCO_3. Fractions containing the asialo glycoprotein were pooled and lyophilized. Mouse GP-66sm was converted to its asialo form with neuraminidase (Mehansho and Carlson, 1983). Deglycosylation decreased the molecular weight of GP-158 and GP-66 by 40% (Mehansho and Carlson, 1983; Mehansho et al., 1985b).

Turbidimetry Measurements. The turbidity produced by tannin/GP-66sm interactions in distilled H_2O was monitored by measuring the absorbance at 500 nm. The initial concentration of both tannin and GP-66sm was 0.03 mg/mL in H_2O, giving a 1:1 weight ratio. Aliquots of tannin solution were then added until a 10:1 weight ratio of tannin to protein was attained. This procedure was used to monitor the turbidity of interactions between tannin and nonglycosylated peptide, between B-9 and GP-66sm, and between B-9 and nonglycosylated peptide.

RESULTS

Affinity of Native, Asialo, and Deglycosylated Rat GP-158 for Tannin. The relative affinities of native, deglycosylated and asialo GP-158 for sorghum and quebracho tannin are presented in Table I. Relative affinity is defined as the amount of labeled standard protein in the assay, divided by the amount of competitor necessary to diminish precipitation of the labeled protein by 50% (Asquith and Butler, 1985). Relative affinity values are the average of independent duplicates, which generally varied by ±3% or less. Table I lists the relative affinities calculated on the basis of moles of protein. Native GP-158 has the highest affinity for either tannin, followed by asialo and then deglycosylated protein.

There is an approximate 2-fold difference in relative affinity between native and asialo proteins. The differences are much more pronounced between native and deglycosylated GP-158. For sorghum tannin, native GP-158 is 4-fold more strongly bound than deglycosylated GP-158 on a mole of protein basis. For quebracho tannin, native GP-158 has 7-fold greater affinity than deglycosylated GP-158 on a mole of protein basis.

Purified oligosaccharides did not inhibit the precipitation of [^{14}C]BSA by either tannin (Table I). This procedure detects formation of soluble complexes with tannins, as well as precipitates. A 7-fold molar excess of oligosaccharides over labeled BSA did not change the amount of [^{14}C]BSA in the pellet. This indicates that the isolated oligosaccharides have little or no affinity for the tannins, although the carbohydrate on the native glycoprotein enhances the affinity for tannins.

Quebracho tannin has a 2-fold higher affinity for native and asialo GP-158 than does sorghum tannin (Table I). Removing sialic acid causes the affinity of the protein for both tannins to decrease by roughly 50%, but the asialo protein retains an almost 2-fold higher affinity for quebracho tannin. However, deglycosylated GP-158 has the same affinity for both tannins. Removal of the entire chain causes the proteins' affinity for quebracho to decrease 7.5-fold as opposed to a smaller decrease of only 4-fold for sorghum tannin. The oligosaccharides not only increase

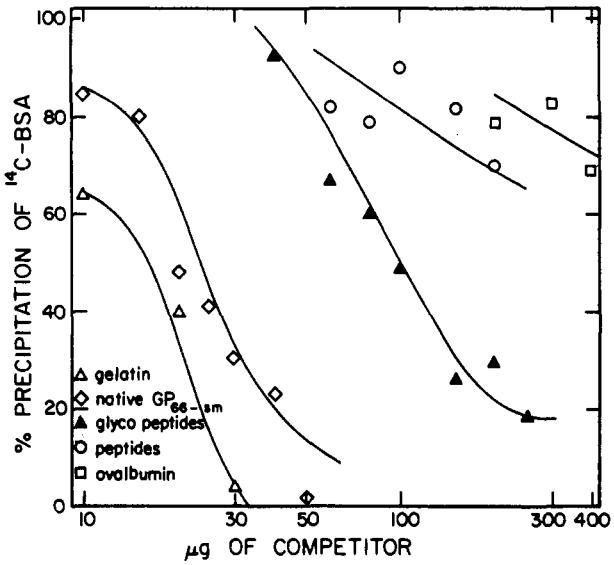

Figure 1. Relative affinity values of glycosylated or nonglycosylated peptides from GP-66sm, or the native protein, determined for sorghum tannin. Gelatin and ovalbumin were utilized as standards. The peptides or proteins were individually competed against labeled BSA for binding to tannin as outlined in the text.

the affinity of these proteins for tannin but also confer selectivity on the binding of the protein to tannin.

Tannin Binding by Nonglycosylated Peptides vs. Glycosylated Peptides. Peptides and glycopeptides generated from mouse GP-66sm by clostripain treatment differ from each other in their amino acid sequences primarily where threonine replaces proline (Clements et al., 1985). The threonine residues are the sites of oligosaccharide attachment in the glycosylated peptides. The two peptide families and native GP-66sm were individually tested in the competition assays against [^{14}C]BSA for binding to sorghum tannin (Figure 1). In addition, gelatin and ovalbumin were utilized as "high-affinity" and "low-affinity" standards (Hagerman and Butler, 1981). The results are plotted on a semilog scale to accommodate the wide range in competitor concentrations. The relative affinities of competitors decrease as they fall further to the right on the figure.

The glycopeptides have 6-fold greater relative affinities for tannin than do the nonglycosylated peptides, thus indicating that the carbohydrate moiety contributes substantially to the binding of the peptides to tannin. The difference in affinities between peptides and glycopeptides is 12-fold on a mole of peptide basis, because the glycopeptides are 50% sugar by weight (Mehansho et al., 1985a). The affinity of the nonglycosylated peptides is equivalent to that of a 13-peptide of polyproline (Hagerman and Butler, 1981). Native GP-66sm is roughly 4 times more effective than its glycopeptides at inhibiting the precipitation of [^{14}C]BSA by tannin.

Removal of Sialic Acid from Mouse GP-66sm. Treatment of native GP-66sm with sialidase to remove terminal sialic acid residues caused the relative affinity to decrease by 50% (Figure 2). This is consistent with the results for the affinities of rat native and asialo GP-158 for tannin (Table I).

Turbidimetry of Complexes between Mouse GP-66sm or Peptides and Tannin Trimer or Polymers. Soluble complexes formed by GP-66sm and B-9, GP-66sm and tannin, and nonglycosylated peptide and B-9 showed minimal absorbance (Figure 3). However, complexes formed by nonglycosylated peptide and tannin showed increasing absorbance as the tannin concentration was

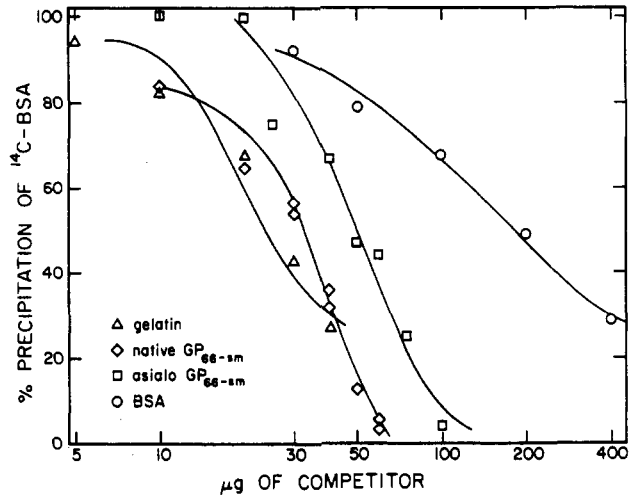

Figure 2. Relative affinity values of native or asialo GP-66sm determined for sorghum tannin. Sialic acid was removed with *C. perfringens* neuraminidase as described in the text. Gelatin and BSA were tested as standards. The proteins were individually competed against labeled BSA for binding to tannin as outlined in the text.

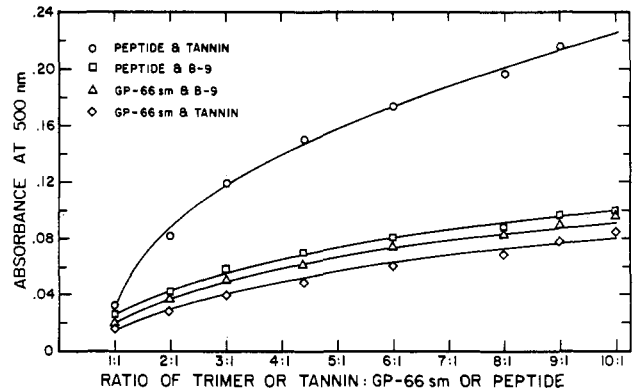

Figure 3. Turbidity produced by four complexes monitored as the ratio of trimer (B-9) or tannin to GP-66sm or nonglycosylated peptide was increased. Turbidity was measured as absorbance at 500 nm. The concentration of all components in a solution with a 1:1 ratio was 0.03 mg/mL of H_2O. Aliquots of the trimer or tannin were added to increase the ratio up to 10:1.

raised. At a ratio of 10:1 (tannin to nonglycosylated peptide) precipitation occurred. No precipitate was observed with any of the other complexes. This suggests that the solubility of the tannin complexes with glycosylated peptides is greater than that of the complexes with nonglycosylated peptides. HPLC analysis of the supernatant layer from tannin/nonglycosylated peptide precipitations showed that the longest tannin polymers had been selectively removed from solution (Putman, 1987).

DISCUSSION

Sorghum and quebracho tannins have very different affinities for native and deglycosylated GP-158. However, they have similar affinities for many other proteins (Asquith and Butler, 1986) despite differences in the structures of these tannins (Viviers et al., 1983; Delcour et al., 1983). This phenomenon is related to the oligosaccharide component of the glycoprotein because deglycosylated GP-158 has the same affinity for both tannins (Table I).

Proteins that have open, loose conformations and relatively high molecular weights efficiently bind to tannin because the two polymers can freely interact (Hagerman and Butler, 1981). Salivary PRPs fit these criteria due to their size, conformational mobility, high content of proline, and random-coil structures (Muenzer et al., 1979). Re-

cently, McManus et al. (1985) noted a strong correlation between binding affinities for BSA and conformational mobilities of tannic acids.

The importance of oligosaccharides to the conformation of hydroxyproline-rich glycoproteins has been demonstrated (van Holst and Varner, 1984; Stafstrom and Staehelin, 1986). In both cases removal of the carbohydrate caused the proteins to assume more compact conformations. We propose that the oligosaccharides of glycosylated PRPs enhance tannin binding by maintaining the proteins in relatively open conformations. Molecular models of mouse GP-66sm glycopeptides indicate that steric interference between the oligosaccharides and the three sequential proline residues (Clements et al., 1985) is minimized when the polypeptide chain is in an extended rather than compact conformation. Similar effects are probably involved in the different tannin affinities between native and deglycosylated GP-158. Alkaline PRPs, which have comparable amino acid compositions but no carbohydrate (Muenzer et al., 1979), have lower relative affinity values for tannin than GP-158 or GP-66sm (Asquith, 1985).

An extended, open conformation of the protein would be more accessible for formation of hydrogen bonds with tannin (Hagerman and Butler, 1981). The observation that removing sialic acid decreases PRP affinity for tannin is consistent with this model. Electrostatic repulsion between sialic acid residues would maintain the protein in a more open conformation.

The turbidity data indicate that tannin/peptide complexes are much less soluble than tannin/protein or trimer/peptide complexes. This suggests that the solubility of polyphenol/protein complexes depends on the physical properties of the ligands (size, conformation, chemical substitution, etc.). Sugar moieties may enhance the solubilities of PRP/tannin complexes. Jones and Mangan (1977) noted that bovine submaxillary mucin was not precipitated by tannin, suggesting that it may bind tannin but remain soluble. Soluble tannin/protein complexes may play a significant role in overcoming the anti-nutritional effects of dietary tannin.

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