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Received for review October 11, 1976. Accepted August 20, 1977. This work was supported by a Grant-in-Aid from the TransFRESH Corporation, Salinas, Calif.

Interactions of Globular Protein with Simple Polyphenols

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Isolated arachin (the major protein in seeds of the genus *Arachis*) was exposed to concentrations of catechol and pyrogallol ranging from 0.05 to 0.3 M. After removal of these phenols by dialysis, conformational modes of arachin, determined by circular dichroism, indicated that native arachin increased in α-helical content. Exposure to high concentrations of catechol and pyrogallol also changed the antigenic reaction of native arachin with antiarachin from one of identity to one of partial identity. In vitro pepsin hydrolysis showed that arachin exposed to 0.1 M catechol or pyrogallol was more susceptible to hydrolysis than was hydrolysis of native arachin. These data on a model system (arachin) describe the effects of phenols on protein digestibility, solubility, and conformation.

The interactions of phenols or their oxidation products with proteins have long been known. These interactions range from inhibition and activation of enzymes to the formation of certain tastes and flavors in foods (Bate-Smith, 1973; Goldstein and Swain, 1965). As pointed out by Van Sumere et al. (1975), the chemistry and biochemistry involved in these reactions are far from being understood. Some complex polyphenols such as tannins are present in many cereal foods, e.g., grain sorghum, and are reputed to inhibit protein digestibility (Nelson et al., 1975). Tannins are categorized into two groups—condensed and hydrolyzable—and according to Loomis and Battaile (1966), they react differently with polypeptides. The numerous possible reactions between phenols and proteins were reviewed by Van Sumere et al. (1975).

The present study relates to interactions in a model system between a purified protein and simple polyphenols that are basic units of hydrolyzable tannins (Loomis and Battaile, 1966). The analyses are intended to establish a correlation between molecular conformation of protein and susceptibility to enzyme hydrolysis (digestibility). The fairly well characterized peanut globular protein, arachin, was chosen as the model protein (Jacks et al., 1975; Shetty and Rao, 1976). Structural measurements were made with circular dichroic spectra and immunochemistry, and relative digestibilities were determined by pepsin hydrolysis in vitro.

EXPERIMENTAL SECTION

Treatment of Protein. Arachin (50 mg), isolated according to Neucere (1969), was dissolved in 4-mL portions of distilled water, pH 5.5, that contained from 0.05 to 0.3 M catechol or pyrogallol plus a control. Some precipitation was observed for all of the samples including the control. The control and the treated samples were then dialyzed (molecular weight cutoff 6000 to 8000) against phosphate buffer, pH 7.9, ionic strength 0.2, for 24 h at 5 °C to remove the phenols. After dialysis, all samples were centrifuged at 40 000*g* for 20 min, yielding clear supernatants; these were used to assess differences in protein solubility and digestibility and for immunochemical determinations. For analysis by circular dichroism, separate samples were treated with 0.2 M aqueous catechol or pyrogallol and then dialyzed against distilled water instead of phosphate buffer because the phosphate ion interferes with the analytical procedure. These samples were subsequently freeze-dried for later spectral determinations with aqueous NaF as the optically clear solvent.

Enzymatic Digestion In vitro. Digestion of treated and untreated arachin was performed in duplicate according to Mauron (1971), with some modifications. Portions of 1.5 mL from the samples dialyzed against phosphate buffer that contained 18.75 mg of protein were made to 0.1 N H_2SO_4. Each sample was then treated with 5 mg of B grade pepsin (Calbiochem) and digested in closed test tubes at 37 °C for 24 h. After precipitation of undigested protein with 70% ethanol followed by centrifugation, the supernatants were analyzed for free amino groups.

Analytical Methods. For immunochemical analyses and assessment of solubility differences, protein contents were determined by the Kjeldahl method or by the method of Lowry et al. (1951). Semiquantitative analyses of treated and untreated arachin by electroimmunodiffusion were performed according to the method of Laurell (1966); sample wells contained 10.0 μg of protein for electrophoresis in agar that contained 1% antiserum against arachin. The antiserum was prepared by Antibodies Incorporated, Davis, Calif. Electrophoresis proceeded for 15 h at 150 V and 7.0 ma. Qualitative analyses of treated and untreated arachin were performed by double diffusion in agar according to Ouchterlony (1949). Free amino groups were determined according to Clark (1964) whereby glycine, expressed in mass units, was used as a standard. Blanks containing catechol or pyrogallol showed only trace absorbances. Circular dichroic spectra of freeze-dried protein samples dissolved in 0.3 M aqueous NaF were obtained with a Cary Model 60 spectropolarimeter equipped with a 6001 CD attachment. Amounts of α-helical, pleated sheet, and unordered conformational modes in each protein sample were calculated by com-

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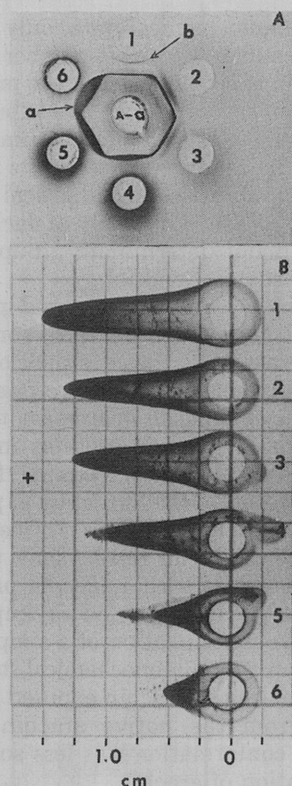

Figure 1. Double diffusion in agar, A, and antibody-in-gel electrophoresis, B, of arachin treated with different concentrations of catechol in phosphate buffer, pH 7.9, ionic strength 0.2. Wells 1, 2, 3, 4, 5, and 6 correspond to samples that contained no catechol (controls), 0.05, 0.1, 0.15, 0.20, and 0.25 mol of catechol, respectively. A-a refers to antiarachin. Antigen wells A and B each contained 50 and 10 μg of protein, respectively.

paring ellipticity values for the protein with values for conformationally known polypeptides at several wavelengths, as determined and described by Greenfield and Fasman (1969). Protein concentrations were determined from absorbances of the solutions at 280 and 260 nm (Clark, 1964).

RESULTS AND DISCUSSION

The effects after exposure of different concentrations of catechol on the antigenic reaction of arachin with antiarachin are shown in Figure 1. Qualitative analysis by double diffusion in part A shows that the nature of the precipitin reaction gradually changes after exposure to increasing concentrations of catechol. The diffused reaction that occurred after high concentrations (arrow a) of catechol indicated a reaction partially identical with that of the control. The site of a precipitate depends on the initial concentrations of the reactants and their diffusion coefficients. If the antigen has determinants that have separate specificities (perhaps on nonidentical protein subunits and/or within their secondary structure), then such a deviated reaction is expected. A previous study by Jacks et al. (1975) provided evidence that antibodies to native arachin involved conformational determinants. Note that a minor protein present in the arachin preparation (arrow b) was no longer antigenic after exposure to 0.15 M catechol (well 3).

Semiquantitative analysis in part B (Figure 1) is based on the electrophoretic migration of a protein in agar that contains its antibody. The length of each conical peak (antigen–antibody complex) is directly proportional to concentration, provided the protein has the same net charge and degree of antigenic specificity (or the same number of determinant groups). These parameters,

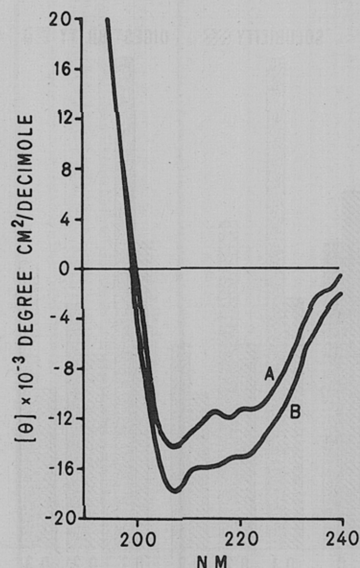

Figure 2. Far ultraviolet circular dichroic spectrum of arachin after exposure to 0.2 M pyrogallol (A) and 0.2 M catechol (B). Protein concentration was 0.16 mg/mL for catechol-treated protein and 0.11 mg/mL for pyrogallol-treated protein.

Table I. Contents of α-Helical, Pleated Sheet, and Unordered Structures in Conformation of Treated and Untreated Arachin

	Conformational modes[a]		
Treatment	α-Helical	Pleated sheet	Unordered
Control[b]	14.6	27.0	58.4
0.2 M pyrogallol	31.9	18.8	49.3
0.2 M catechol	45.5	13.4	41.1

[a] Values were computed from circular dichroic spectra as described in the text and are given as percentages of the total conformation. The closeness of fit of each spectrum to the theoretical spectrum for each protein of the conformational modes given above is estimated by the following standard deviations ($[\theta] \times 10^{-3}$ degree cm/dmol): pyrogallol treated, ±0.61; catechol treated, ±0.77.
[b] From Jacks et al. (1975).

however, can vary with change in molecular conformation. The results showed a gradual decrease in the migration of arachin after exposure to increased concentrations of catechol. Judging from the intensity of staining and the length of the conical peaks, it appeared that treated arachin gradually changed in antigenic specificity after exposure to high concentrations of catechol. Again, as shown by double diffusion, there is evidence of some type of conformational change induced by catechol. After exposure of arachin with identical concentrations of pyrogallol, similar results were obtained.

Figure 2 shows the circular dichroic spectra of samples of arachin exposed to pyrogallol (A) and with catechol (B). Calculations of the conformational modes from the spectra (Table I) indicated a drastic increase in α-helical content that was much greater for catechol than for pyrogallol. Undoubtedly, the reaction must be of hydrogen bond type, either through interpeptide chains or encompassing the phenols themselves. Perhaps the difference in α-helical induction between catechol and pyrogallol could be attributed to the number of hydroxyl groups on each phenol.

The solubility of a protein is a function of many factors. These include molecular conformation, amino acid composition, ionic strength of the solvent, and the extent of denaturation that might have occurred. After exposure to three different concentrations of catechol and pyrogallol,

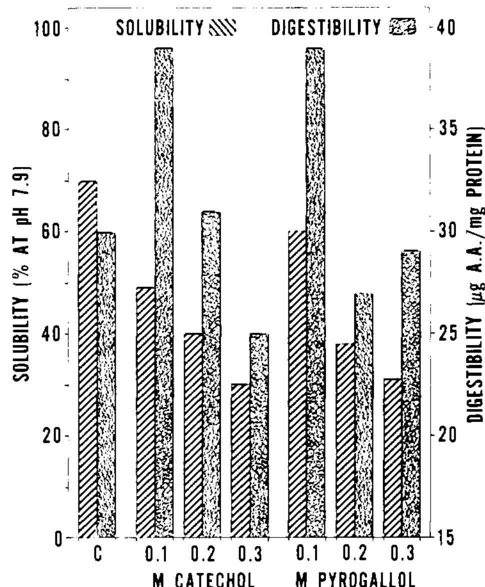

Figure 3. Differences in solubilities expressed as percent of the original material (12.5 mg/mL) and digestibilities of arachin after exposure to different molarities of catechol and pyrogallol.

reductions in solubilities of arachin were induced, as shown in Figure 3. The effects were less prominant in samples exposed to 0.1 M pyrogallol than to 0.1 M catechol and were about the same for proteins exposed to 0.2 M and 0.3 M concentrations of the phenols.

Relative protein digestibility of arachin determined by in vitro enzyme hydrolysis using pepsin is also shown in Figure 3. Although the extent of hydrolysis was small in all of the samples, the quantity of amino acids released was slightly greater in samples that were exposed to 0.1 M catechol or pyrogallol. Perhaps the number of peptide linkages in arachin susceptible to hydrolysis increased at this concentration of polyphenols. If so, some of these peptide linkages would have to be exposed from the interior of the molecule since pepsin is an endoaminopeptidase. The digestibilities at 0.2 and 0.3 M concentrations of the phenols were almost the same as that of the control.

These data provide evidence that exposure of certain concentrations of catechol and pyrogallol to a globular protein induced the formation of α-helical structure. The reactions could involve either interpeptide hydrogen bonding as a result of conformation changes, or perhaps hydrogen bonding through the hydroxyls of the phenolic compounds. Using a model system, Anderson and Sowers (1968) described the hydrogen bonding of plant phenols to polyvinylpyrrolidone. In another study, Loomis and Battaile (1966) showed that phenols were removed from hydrogen bonded complexes with protein by adding large amounts of polyvinylpyrrolidone. Certainly other reactions from possible quinone formation could be involved, but hydrogen bonding seems to be most plausible in view of the spectral data.

As for the immunochemical data, only qualitative interpretation is possible. The exact number of determinant groups on arachin is not known, but the protein is known to be comprised of several associative subunits (Neucere, 1969; Tombs, 1965). Electrophoretic/immunochemical data indicated that some of these units have different electrophoretic mobilities but identical antigenicities (Daussant et al., 1969). The study of Jacks et al. (1975) found that the major determinant groups were conformational rather than sequential.

The presence of plant phenols and polyphenols in general is significant when the nutritional statuses of certain foods are assessed. Besides their obvious adverse effects such as producing allergenic responses in humans and inducing loss of appetite in animals, they are known to inhibit the digestibility of proteins in vivo, thereby reducing the nutritional value of foods and feeds. Whether this effect was a result of the formation of phenol–protein complexes and/or simple inhibition of certain digestive enzymes is not certain. Our results show that for simple polyphenols interacting with a specific protein in vitro, digestibility was either enhanced or impaired slightly depending on the concentration of a simple polyphenol. Circular dichroism and immunochemical studies indicated that the conformation of arachin exposed to polyphenols was different from the native arachin. This polyphenol-induced conformation was less soluble than the native conformation of arachin.

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Received for review April 11, 1977. Accepted September 7, 1977. Use of a company or product name by the U.S. Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.