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Interaction of Synthetic Proanthocyanidin Dimer and Trimer with Bovine Serum Albumin and Purified Bean Globulin Fraction G-1

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To evaluate the type of binding involved, thermodynamic analysis of the temperature dependence of proanthocyanidin binding to bovine serum albumin (BSA) and bean glycoprotein G-1 (G-1) was investigated. Binding was analyzed with tritiated proanthocyanidin by ultrafiltration to separate free ligand and protein-bound ligand. Binding constants were determined from Scatchard plots. Van't Hoff plots indicated proanthocyanidin binding to BSA was spontaneous and entropy driven. Analysis with *cis*-parinaric acid supported the conclusion drawn from the thermodynamic analysis that the binding of proanthocyanidin to BSA was a hydrophobic interaction. Van't Hoff plots indicated proanthocyanidin binding to native G-1 protein was also spontaneous but, in contrast to BSA, enthalpy driven. Analysis with *cis*-parinaric acid confirmed the hydrophilic character of proanthocyanidin binding to native G-1. Evaluation of proanthocyanidin binding to heat-denatured G-1 with *cis*-parinaric acid indicated hydrophobic interactions.

Proanthocyanidins or condensed tannins are polymeric plant phenolics that interact with protein, decreasing protein digestibility (Feeny, 1969; Aw and Swanson, 1985). The mechanism of the interaction has received substantial attention, and several mechanisms have been proposed. The two major noncovalent binding mechanisms proposed were hydrogen bonding and hydrophobic interactions. Gustavson (1956), Loomis and Battaile (1966), Loomis (1974), and van Sumere et al. (1975) have proposed hydrogen bonding as the probable mechanism. Loomis and Battaile (1966) concluded on the basis of the interaction of condensed tannin with polymers like polyvinylpyrrolidone (PVP), gelatin, collagen, and nylon that the mechanism was hydrogen bonding. Haslam (1974) evaluated the association of proanthocyanidin dimer and trimer with glucosidase. He concluded that hydrogen bonding occurred between phenolic hydroxyl groups and peptide bond carbonyl groups. Hagerman and Butler (1981) enhanced hydrogen bonding of condensed tannin to formamide by alkyl substitution on the amide N adjacent to the carbonyl. Formamide had less affinity for condensed tannin than the substituted derivatives *N*-methylformamide or *N,N*-dimethylformamide. Electron-donating methyl groups on the N adjacent to the carbonyl increased the electronegativity of the oxygen on the carbonyl group. They suggested this increased the strength of the hydrogen bonds between the carbonyl and the proanthocyanidin hydroxyls.

There is also evidence of hydrophobic interaction between proanthocyanidin and protein. Detergents (Goldstein and Swain, 1965) as well as organic solvents (Loomis, 1969) can partially dissociate tannin from protein. Calderon et al. (1968) found ethanol decreased the affinity of tannin for protein while NaCl increased the affinity of tannin for protein. Oh et al. (1980) indicated that the predominant mode of interaction was hydrophobic based on several observations: (1) protein was eluted from a column of immobilized tannin with detergents; (2) interaction between tannin and protein was enhanced with increased ionic strength and temperature; (3) tannins were absorbed readily onto uncharged polystyrene columns. Butler et al. (1984) suggested that interactions between tannin and protein involve hydrogen bonding and hydrophobic associations.

The type of interaction has not been completely elucidated. Thermodynamic analysis provides a means for rigorously determining the type(s) of interactions involved. Hydrophobic interactions are also called entropy-driven interactions because they are characterized by a positive change in entropy and enthalpy. On the other hand, hydrophilic interactions are characterized by a decrease in enthalpy. To obtain estimates for the entropy and enthalpy changes upon binding, Van't Hoff plots are often

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used. The slope of the plot provides the enthalpic contribution while the intercept gives the entropic contribution (Lauffer, 1975).

MATERIALS AND METHODS

Proanthocyanidin Synthesis. All chemicals used were reagent grade unless otherwise specified. *cis*-Parinaric acid (9,11,13,15-*cis*,*trans*,*trans*,*cis*-octadecatetraenoic acid) was purchased from Molecular Probes, Inc. (Junction City, OR), dihydroquercetin from Pfaltz and Bauer, Inc. (Waterbury, CT), and tritiated sodium borohydride from New England Nuclear (Boston, MA). Amberlite XAD-1 (XAD-1) was obtained from Mallinckrodt (Paris, KY), polyvinylpyrrolidone (PVP) from Aldrich (Milwaukee, WI), and Sephadex LH-20 from Pharmacia (Piscataway, NJ).

Dimeric and trimeric proanthocyanidin were prepared by the reduction of dihydroquercetin with sodium borohydride in the presence of recrystallized catechin (3×) under an atmosphere of nitrogen (Eastmond, 1974). The polymerization of dihydroquercetin and catechin was followed with silica gel thin-layer chromatography (TLC) using an acetone–toluene–formic acid (60:30:10) solvent (Lea, 1978). Tritium-labeled catechin dimer and trimer were prepared with tritiated sodium borohydride in the same manner as with unlabeled sodium borohydride. Both labeled and unlabeled proanthocyanidin dimer and trimer were separated on a column of Sephadex LH-20 with an ethanol–water (45:55) solvent. The eluent was monitored continuously at 280 nm with a Model SC-15 ISCO UV monitor (Lincolin, NE). Radioactivity of each labeled fraction was determined with a scintillation counter (Packard Model TRI-CARB 460CD, Downers Grove, IL). Fractions containing each oligomer were collected, combined, rotoevaporated to remove ethanol, and then freeze-dried. Elution order was monomer, dimer, and then trimer. There was an increase in radioactivity corresponding to an increase in absorbance as each proanthocyanidin oligomer eluted from the column. Purity of the dimer and trimer fractions was monitored with silica gel TLC (Lea, 1978). Fractions containing a second discernible spot from the TLC analysis were collected, the solvent was removed, and the material was rechromatographed with Sephadex LH-20. To determine the purity of the oligomers, a Waters Associates high-performance liquid chromatograph (HPLC) (Milford, MA) equipped with two Model 6000 A pumps and a variable-wavelength detector set at 279 nm (Perkin-Elmer Model LC 75, Norwalk, CT) was used. A reversed-phase column (Microsorb ODS, 3 μm, E. Merck & Co.) was used. Acetic acid in water (4% v/v) was the eluent (Mulkay et al., 1981). Purity, as determined by HPLC, was greater than 97.0% for both the dimer and trimer.

Eastmond (1974) identified dimeric and trimeric catechin as the reaction products from the reduction of dihydroquercetin with sodium borohydride in the presence of catechin with paper chromatography, UV spectroscopy, gas–liquid chromatography, and mass spectroscopy. According to Eastmond (1974) only dimeric and trimeric oligomers containing C_4–C_8 carbon bonds were produced during the synthesis. Gradient elution with acetic acid and water according to the method of Jerumanis (1985) indicated only one major peak with both the dimer and trimer fractions.

Protein Purification and Preparation. Bovine serum albumin (BSA) was used since it is capable of hydrophobic binding, its primary function being lipid transport in the blood. Purified defatted BSA was purchased from Sigma Chemical Corp. (St. Louis, MO). The bean (*Phaseolus vulgaris* L.) glycoprotein G-1, the major storage protein in beans (Romero and Ryan, 1978), was used since it exists in legumes along with proanthocyanidin in colored legume seeds. G-1 was purified according to the method of Stockman et al. (1976), based on the reversible pH-dependent polymerization of G-1, with slight modifications. XAD-1 and PVP (3.5 g each) were added to 7 g of the white, dehulled bean flour to bind any free phenolic compounds. To extract the phenolics, the bean flour, plus XAD-1 and PVP, was washed at 21 °C for 5 min with 150 mL of acetone–water (80:20, v/v) containing 0.1% (w/v) ascorbic acid in a 400-mL beaker with continuous stirring. The flour was washed for 5 min in a Buchner funnel with a second aliquot (350 mL) of the same acetone–water solvent. Next, G-1 was extracted at 21 °C for 30 min with 100 mL of 0.5 M NaCl, pH 8.5, 0.1 M bicarbonate buffer in a 400-mL beaker with continuous stirring. The G-1 was purified by affinity chromatography (Stockman et al., 1976). Discontinuous SDS slab gel electrophoresis [Laemmli as modified by Stockman et al. (1976)] indicated only three distinct bands corresponding to the three subunits of G-1 (Stockman et al., 1976). The G-1 isolate showed no trace bands of either the other major globulin or other impurities. After purification, G-1 was desalted and concentrated with an Amicon pressure filtration system with an Amicon PM-30 filter at 2 °C. After desalting, the G-1 was freeze-dried and stored at −20 °C.

Partially denatured G-1 (2.0 mL, 0.08% w/v, in 0.2 M NaCl, pH 7.0, 0.01 M phosphate) was prepared by heating in boiling water for 10 min in capped 25-mL test tubes. The test tubes were cooled in ice water immediately after heating. No precipitation of G-1 was observed.

Binding Analysis. Binding constants of ligand (proanthocyanidin dimer and trimer) to BSA and G-1 were determined by the method of Sophianopoulos et al. (1978) with an Amicon Micropartition System MPS-1 (MPS-1). Binding was determined at pH 7.0 to maintain G-1 solubility, to keep G-1 in a monomeric form (G-1 polymerizes below pH 6.5), and to evaluate the interaction near the physiological pH of beans. Separation of the free ligand from the bound ligand was accomplished by convective filtration of free ligand through an anisotropic, hydrophilic YMT ultrafiltration membrane (Amicon Corp., Danvers, MA). Protein and protein-bound ligand were quantitatively retained above the membrane while the low molecular weight ligand filtered through.

Proanthocyanidin dimer concentrations were 23.89–1531.0 μg/mL in distilled water, while trimer concentrations were 9.3–111.6 μg/mL in distilled water for the BSA-binding study. The BSA concentration was 7 mg/mL in the dimer/ligand experiment and 1 mg/mL in the trimer/ligand experiment. For the G-1 and trimer experiment, the G-1 concentration was 1 mg/mL and trimer concentrations were 9.3–111.6 μg/mL in distilled water. BSA and G-1 were prepared in 0.2 M NaCl, pH 7.0, 0.01 M phosphate buffer.

Each MPS-1 and membrane was pretreated with the addition of ligand at the same concentration, specific activity, and temperature as for the respective binding analysis. To determine the efficacy of the pretreatment several aliquots of labeled proanthocyanidin oligomer were added in sequence to the MPS-1 apparatus. The MPS-1 was centrifuged after the addition of each aliquot. A slight reduction in the proanthocyanidin oligomer concentration occurred in the first aliquot added to the MPS-1 containing the membrane. The addition of three more aliquots in sequence showed no increase or decrease in radioactivity, indicating neither additional binding nor release of ligand.

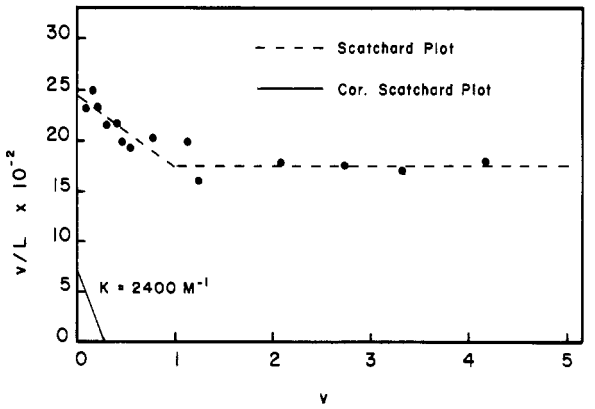

Figure 1. Scatchard plot of BSA and proanthocyanidin at 19 °C.

The pretreatment procedure for both the highest and lowest concentrations used in the binding analysis was evaluated.

Ligand solution (0.6 mL) was added to the protein solution (1.6 mL), mixed, and allowed to equilibrate for 10 min. The three temperatures used in the experiment were 19, 29, and 39 °C. Each analysis was done in triplicate. After equilibration, 0.6 mL of the mixture was added to each MPS-1 and centrifuged (2000*g* for 8, 7, and 6 min, respectively, at 19, 29, and 39 °C). Two replicates (100 μL) from each MPS-1 system were analyzed. The scintillation fluid used was Scinti Verse II (SO-X-12, Fisher Scientific Co.). All ligand concentrations were determined with a scintillation counter (Packard Model TRI-CARB 460CD, Downers Grove, IL). Free ligand concentration was determined from a standard curve. Binding constants were determined by Scatchard plot analysis (Weiland et al., 1979).

Binding constants of BSA and proanthocyanidin dimer and trimer were determined from Scatchard plot analysis for the three different temperatures 19 (Figure 1), 29, and 39 °C. The free ligand concentration is [L], while υ is the moles of ligand bound/mole of protein. The equilibrium binding constant was equal to the negative slope of the corrected Scatchard plot curve.

Linear regression was used to determine the high-affinity binding. The nonspecific binding of the dual-component system was determined and corrected for by the method of Chamness and McGuire (1975) for a two-component system, where one component has a much lower affinity than the other. Nonspecific binding was determined as the *y*-axis intercept of the lower affinity binding line. Chamness and McGuire (1975) state that the ratio of bound ligand to free ligand will reach a limiting value after saturation of the high-affinity sites. The slope of the line corresponding to the limiting value for nonspecific binding is zero (Chamness and McGuire, 1975). To correct for the nonspecific binding, according to the method of Chamness and McGuire (1975), the limiting ratio of υ/[L] is determined and then multiplied by the free-ligand concentration, [L], for each υ to determine nonspecific binding at that point. This is then subtracted from the total binding, υ_{TOTAL}, to find the specific or high-affinity binding υ_{SP}:

$$\upsilon_{SP} = \upsilon_{TOTAL} - [L] \lim_{\upsilon \to \infty} (\upsilon/[L])$$

The υ_{SP} is plotted vs. υ_{SP}/[L] to produce the corrected binding line or corrected Scatchard plot curve.

Fluorescence Analysis. The surface hydrophobicity of a protein can by evaluated with the fluorescent surface probe *cis*-parinaric acid (Nakai et al., 1980; Kato and Nakai, 1980). A large increase in the fluorescence quantum yield of *cis*-parinaric acid occurs when *cis*-parinaric acid partitions from a polar environment into a nonpolar environment, e.g. from an aqueous solvent to the lipid binding domain of BSA (Sklar et al., 1977). The method of Kato and Nakai (1980) for determining protein surface hydrophobicity was adapted for evaluating proanthocyanidin dimer binding to BSA and G-1. BSA and G-1 were prepared in buffer (0.2 M NaCl, pH 7.0, 0.01 M phosphate) at four concentrations [0.005%, 0.01%, 0.02%, 0.04% (w/v)]. Procyanidin dimer was prepared in water (25 mg/mL). Aliquots (0–50 μL) of proanthocyanidin were added to two of the four test tubes containing 2 mL of protein solution and then allowed to equilibrate for 10 min. *cis*-Parinaric acid (10 μL, 3.6×10^{-3} M in absolute ethanol) was added to all four test tubes. The fluorescence was determined in an Aminco-Bowman spectrophotofluorometer (Silver Spring, MD) at an excitation wavelength of 325 nm and an emission wavelength of 420 nm. The fluorescence of each individual component was subtracted from the fluorescence of the mixture of protein, proanthocyanidin, and *cis*-parinaric acid at each concentration of added proanthocyanidin. The difference in fluorescence indicated the effect of proanthocyanidin on the binding of *cis*-parinaric acid to BSA and G-1. The equation used to correct for the fluorescence due to each component was

$$F_{CORRECTED} = F_{CP,P,O} - F_{P,O} - (F_{CP,O} - F_O)$$

where $F_{CP,P,O}$ = the fluorescence of the combination of protein, *cis*-parinaric acid, and proanthocyanidin oligomer; $F_{P,O}$ = the fluorescence of protein and proanthocyanidin oligomer; $F_{CP,O}$ = the fluorescence of *cis*-parinaric acid and proanthocyanidin oligomer; and F_O = the fluorescence of proanthocyanidin oligomer.

Table I. Equilibrium Association Constants for Protein and Proanthocyanidin Oligomers

	19 °C	29 °C	39 °C
BSA × dimer	2400[a] ± 440[b]	3100 ± 430	4100 ± 920
BSA × trimer	48000 ± 4000	66000 ± 4000	83000 ± 3000
G-1 × trimer	68000 ± 5000	48000 ± 4000	17000 ± 3000

[a] Units M^{-1}. [b] Standard deviation of regression.

RESULTS AND DISCUSSION

Proanthocyanidin Binding to BSA. Binding constants of BSA and proanthocyanidin dimer and trimer were determined from Scatchard plot analysis for three different temperatures (19, 29, 39 °C) and are given in Table I. Figure 1 is the Scatchard plot for BSA and proanthocyanidin dimer at 19 °C. Thermodynamic analysis of the binding constants of BSA and proanthocyanidin dimer indicated a temperature dependence (Figure 2); i.e., the binding constants increased with increased temperature. The binding constants at 19, 29, and 39 °C are, respectively, 2400, 3100, and 4100 M^{-1}.

The integrated Van't Hoff equation (Weiland et al., 1979) is

$$\ln K = -\Delta H/RT + \Delta S/R \quad (1)$$

where the equilibrium binding constant is K, the change in enthalpy is ΔH, the change in entropy is ΔS, R is the gas constant, and T is the absolute temperature. The slope of a plot of $1/T$ vs. ln K is $-\Delta H/R$. The enthalpy change of the binding of proanthocyanidin dimer to BSA was equal to 4.9 kcal/mol. From eq 1 the entropy change was 32.1 eu. The change in free energy (ΔG) can be determined from

$$\Delta G = -RT \ln K \quad (2)$$

and was equal to −4.5 kcal/mol. The van't Hoff plot indicates, therefore, a reaction with a positive entropy

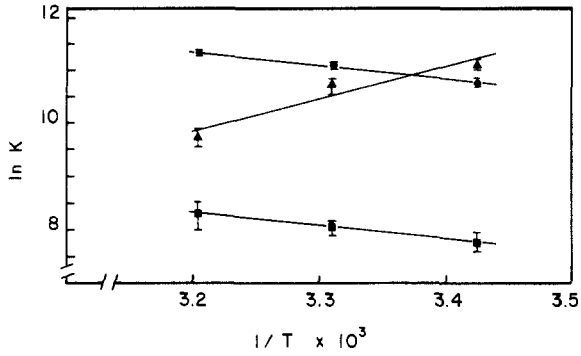

Figure 2. Van't Hoff plots of the dependence of K on temperature. Circles indicate BSA and proanthocyanidin trimer and triangles indicate G-1 and trimer, while squares indicate BSA and dimer. The slope of the line is equal to $-\Delta H/R$. The error bars indicate the standard deviation of regression as determined from the statistical analysis of the slope of the Scatchard plots.

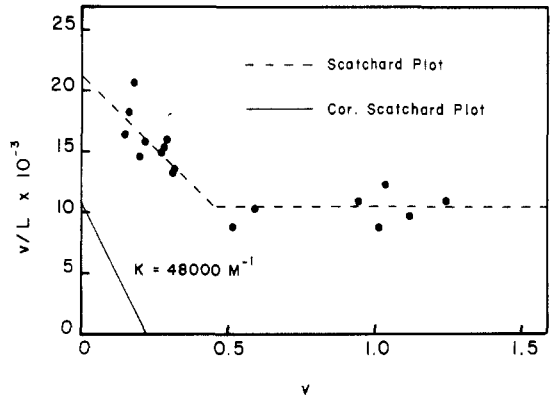

Figure 3. Scatchard plot of BSA and proanthocyanidin trimer at 19 °C.

change, a positive enthalpy change, and a negative free energy change, i.e., a spontaneous reaction that is totally entropy driven. This indicates the binding of BSA to proanthocyanidin dimer is hydrophobic in nature (Weiland et al., 1979).

Scatchard plots of the binding of proanthocyanidin trimer to BSA were done for the 19 (Figure 3), 29, and 39 °C analyses. The equilibrium binding constants were 48 000, 66 000, and 83 000 M^{-1}, respectively. As with the dimer, thermodynamic analysis indicated binding of proanthocyanidin trimer to BSA was temperature dependent (Figure 2). Increased reaction temperature resulted in an increased binding constant. The enthalpy change was equal to 5.0 kcal/mol, ΔS was equal to 38.4 eu, and ΔG was equal to −6.3 kcal/mol. The negative free energy change and positive change in both entropy and enthalpy indicated the binding of proanthocyanidin trimer to BSA was spontaneous and hydrophobic in nature.

The binding of proanthocyanidin dimer and trimer to BSA was driven by the increase in entropy that resulted when water molecules, ordered around the nonpolar ligand binding site on BSA, were displaced (Weiland et al., 1979). Therefore, the driving force for the hydrophobic interaction was the increase in entropy when the ordered water was released to the bulk water. The term entropic union rather than hydrophobic bond might be a better description of the actual situation (Lauffer, 1975).

Proanthocyanidin trimer binds more tightly to BSA than the dimer. Previous work by Haslam (1974) suggested this also. He found that less proanthocyanidin trimer than dimer was needed to precipitate and inactivate amylase.

Previous investigators have observed that *cis*-parinaric acid, an 18-carbon polyunsaturated fatty acid, binds to

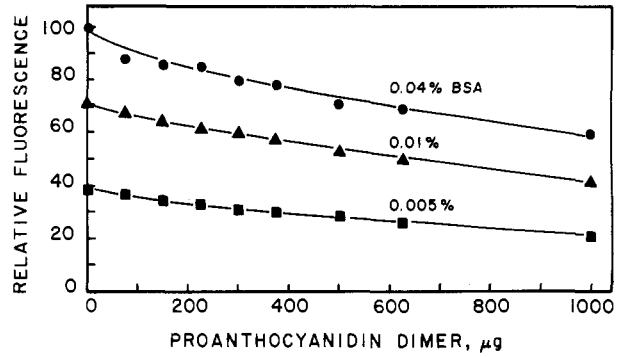

Figure 4. Fluorescence of BSA, proanthocyanidin dimer, and *cis*-parinaric acid.

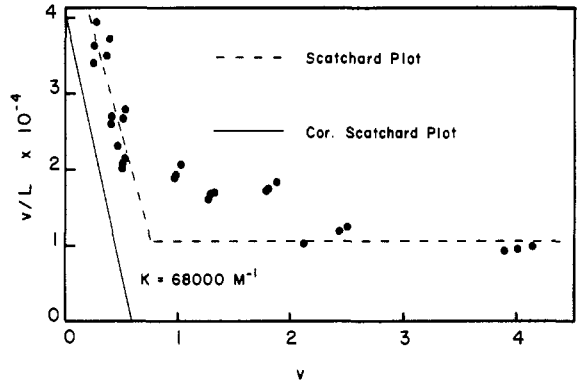

Figure 5. Scatchard plot of G-1 and proanthocyanidin trimer at 19 °C.

serum albumin similar to other 18-carbon fatty acids with respect to mole ratio, binding strength, and specificity (Sklar et al., 1977; Berde et al., 1979; Hsia and Kwan, 1981). The binding has also been characterized as a hydrophobic association with entropy as the major force (Berde et al., 1979).

The fluorescence of *cis*-parinaric acid was reduced as more proanthocyanidin dimer (Figure 4) or trimer was added to BSA prior to the addition of *cis*-parinaric acid. This suggests that proanthocyanidin competes with the hydrophobic compound, *cis*-parinaric acid, for the binding site on BSA, so the interaction is likely to be hydrophobic. Although proanthocyanidin may be hydrogen bonding to the protein and sterically hindering the *cis*-parinaric acid from reaching the site, thermodynamic analysis indicates that proanthocyanidin was bound to the hydrophobic domain of BSA. The results from the fluorescent analysis support the thermodynamic analysis that indicates the binding of proanthocyanidin dimer and trimer to BSA is hydrophobic, rather than hydrophilic, in character.

Proanthocyanidin Binding to G-1. The binding of proanthocyanidin trimer to the bean glycoprotein was also evaluated at temperatures of 19, 29, and 39 °C. Scatchard plots (Figure 5) were used to determine the binding constants of the proanthocyanidin trimer to G-1. The binding constants were 68 000, 48 000, and 17 000 M^{-1}, respectively, for 19, 29, and 39 °C. The binding constant of proanthocyanidin trimer binding to G-1 decreases with an increase in temperature. The enthalpy change was −12.5 kcal/mol and the entropy change was −20.8 eu, while the free energy change was −6.5 kcal/mol, based on the Van't Hoff plot. Proanthocyanidin trimer binding to G-1 is spontaneous and hydrophilic. The binding is driven by the negative enthalpy, which, along with the negative entropy, indicates hydrophilic interactions, van der Waals interactions, and/or hydrogen bonding (Ross and Subramanian, 1981).

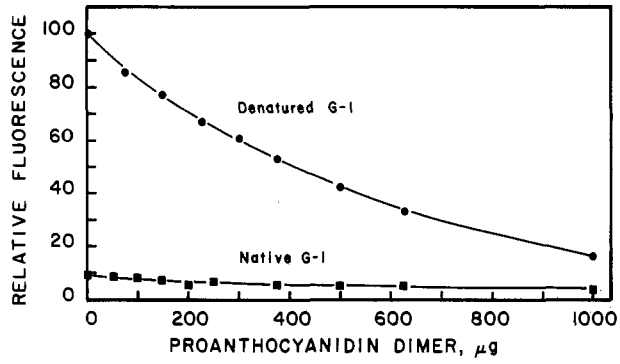

Figure 6. Fluorescence of G-1 (0.04%), proanthocyanidin dimer, and *cis*-parinaric acid.

Heat-denatured G-1 had a surface hydrophobicity greater than that of native G-1. The increase was not unexpected since hydrophobic groups are usually oriented toward the center of the protein in aqueous solvents. Heat denaturation reorientates the hydrophobic groups and exposes them to the solvent. Fluorescence of *cis*-parinaric acid bound to heat-denatured G-1 decreased considerably with the prior addition of proanthocyanidin dimer (Figure 6). The fluorescence data suggested that the binding of proanthocyanidin to native G-1 is different from proanthocyanidin binding to denatured G-1. The effect of proanthocyanidin addition on G-1 (denatured) and *cis*-parinaric acid fluorescence was similar to that of BSA and *cis*-parinaric acid. Binding of proanthocyanidin dimer to denatured G-1 was probably hydrophobic in character, while the interaction of proanthocyanidin and native G-1 was likely polar or hydrophilic in nature.

Results reveal proanthocyanidin oligomers are capable of both hydrophobic and hydrophilic interactions with protein, which previous researchers have suggested. Hagerman (1980) found that tannin–protein complexes were stabilized by both hydrogen bonds and hydrophobic forces.

CONCLUSIONS

Analysis of the equilibrium binding constants of proanthocyanidin oligomer to BSA with respect to temperature indicates the major driving force for the association is the positive entropy. Fluorescent analysis of the interaction indicates that the proanthocyanidin oligomer competes with the *cis*-parinaric acid for the hydrophobic domain on BSA, which supports the thermodynamic data.

The binding of native G-1 and the proanthocyanidin trimer is an enthalpy-driven reaction. The results from the thermodynamic and fluorescent analysis indicate hydrophilic interactions are favored with native G-1 and the proanthocyanidin dimer. However, fluorescent analysis suggests that hydrophobic interactions are favored with denatured G-1 and the proanthocyanidin dimer. With denatured G-1, nonpolar amino acid R groups, normally oriented internally to the protein, were exposed to the solvent. Therefore, the proanthocyanidin–protein interaction is dependent upon the types of protein sites available, which may be altered when the protein is denatured during heating.

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