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PROTEIN–PROANTHOCYANIDIN INTERACTIONS DURING EXTRACTION OF SCOTS PINE NEEDLES

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Key Word Index—*Pinus sylvestris*; Pinaceae; Scots pine; needle protein; proanthocyanidin; condensed tannins; protein–tannin interaction; hydrogen bonds; hydrophobic interaction.

Abstract—Successful protein extractions from Scots pine needles in different buffers (pH 5–9) with PVP-10 and Tween 80 suggest that both hydrogen bonds and hydrophobic interactions are responsible for the formation of insoluble protein–tannin complexes. Gel filtrations did not permit a separation of tannins from solubilized proteins. Contamination of the protein fractions was attributed to proanthocyanidins, both as such and when attached to the proteins. The ability of the soluble protein–proanthocyanidin complexes to adsorb on both Polyclar AT through hydrogen bonding and on Phenyl-Sepharose CL-4B through hydrophobic interactions suggests an amphiphilic capacity on the part of the proanthocyanidin. The factors causing persistent binding between proteins and proanthocyanidins through hydrophobic interactions are discussed. Although the vanillin test confirmed the presence of high molecular weight tannins, they occurred independently of the proteins.

INTRODUCTION

The extraction of proteins from leaves is hampered in various ways by interactions with accompanying phenolic compounds [1, 2]. Among these compounds are protein-precipitating tannins [3] to which the proanthocyanidins (PA, condensed tannins) generally occurring in gymnosperms [4], belong. Using soluble polyvinylpyrrolidone (PVP-10), a detergent (Tween 80), and various buffers (pH 5–9) in combination with gel filtrations and adsorption experiments on insoluble polyvinylpyrrolidone (Polyclar AT) and Phenyl-Sepharose CL-4B, the extraction and separation of protein and tannins from Scots pine needles has been studied. The results suggest that in addition to hydrogen bonds, hydrophobic interactions also mediate PA-protein precipitation and are responsible for persistent contamination of solubilized proteins by PA.

RESULTS

Effects of pH, PVP-10, and Tween 80 on protein yield

Per cent extractable protein is presented in Table 1 in relation to extraction buffer pH and PVP-10 concentration. Without additional PVP-10, a pH above 7.0 was necessary to substantially increase protein yield. Borate buffer was superior to glycine buffer of the same pH value. By increasing the concentrations of PVP-10, the amounts of extractable protein were also increased except in the case of acetate buffer, where this effect was relatively slight. With borate buffer, the maximum yield was reached at the relatively low concentration of 0.25%. Even with the glycine buffer the maximum effect occurred at a relatively low PVP-10 concentration; however, at the highest PVP-10 concentration, the yield was lower than that obtained by using the borate buffer. With decreasing pH values, the positive effects of PVP-10 were reduced. Thus, the results indicated that the effects of increasing pH values and increasing PVP-10 concentrations were additive. In similar experiments, an increase in the protein yield along with increasing concentrations of Tween 80 was also apparent, but it was not as dependent on pH values (Table 1). The borate buffer also gave the highest yields in these tests.

Distribution of proteins and phenolics in relation to the efficiency of the protein extractions

Gel filtrations were used to isolate the solubilized proteins. The results were initially recorded by the absorbance at 280 nm. High absorbances only appeared in the void fractions after extractions in buffers with PVP-10 or Tween 80, which were efficient in the extraction experiments (cf. Table 1). However, the absorbances were too high to have been caused by protein alone. A minor part of the absorbance could have been due to PVP-10 (M_r 10000) or Tween 80 (M_r 1300), in spite of their low-molecular weights compared to the exclusion limit (30000) of the gel (cf. Fig. 4, A3). To determine if proanthocyanidins (PA) were additional contaminating agents they were analysed as anthocyanidins (absorbance at 530 nm) or reacted with vanillin (measured as μg catechin equivalents). Protein content and the absorbance at 280 nm were simultaneously studied in homogenates prepared in either a plain buffer (Fig. 1, A1-D1), with additional PVP-10 (Fig. 1, A2-D2), or further supplemented with sodium chloride (Fig. 1, A3-D3).

A high absorbance at 280 nm was associated with the solubilization of proteins (Fig. 1, A1-A2, B1-B2). PA also occurred simultaneously in the void fractions (Fig. 1, D2). Even in the absence of proteins, a small amount of PA appeared in the same fractions (Fig. 1, D1). However, the PA-test with vanillin did not show the same relationship

Table 1. Effects of buffer type, pH, PVP-10 and Tween 80 on the extraction of protein from Scots pine needles

Additive	Concentration (% w/v)	Extracted protein (% of fr. wt) Acetate pH 5.0	Phosphate pH 7.0	Glycine pH 9.0	Borate pH 9.0
PVP-10	0.00	0.09	0.01	0.63	1.00
	0.06	0.18	0.18	1.10	1.25
	0.13	0.23	0.47	1.11	1.17
	0.25	0.19	0.75	0.94	1.63
	0.50	0.21	0.55	1.04	1.58
	1.00	0.35	1.03	1.19	1.67
Tween 80	0.00	0.26	0.04	0.63	1.02
	0.06	0.30	1.27	1.16	1.80
	0.13	0.91	1.30	1.36	1.47
	0.25	1.41	1.49	1.64	1.87
	0.50	1.49	1.48	1.43	1.89
	1.00	1.93	1.53	1.80	2.02

(Fig. 1, C1, C2), suggesting a heterogeneous affinity among the PA to the proteins. An inhibitory effect (−17%) on protein yield and the yields in both PA tests occurred when ionic strength was increased (Fig. 1, A3-D3). This result could not be attributed to a slight variation (2%) between the fresh weights of the needles used (Fig. 1, A2-D2 and A3-D3). Similar studies on the protein yield at a higher ionic strength (3 M NaCl) did not show any further changes.

Proanthocyanidin contamination of the protein fractions

The relationship between the absorbance at 280 nm and the protein content was studied using gel filtrations of homogenates prepared in acetate and borate buffers with PVP-10. The results are compiled in Fig. 2. Corresponding data for a pure protein (BSA) showed that only a minor part of the observed absorbance could be attributed to the protein moiety of the needle extracts. The extrapolated absorbance, differing from zero in the absence of protein, can be compared with the occurrence of PA, independent of protein as shown above (Fig. 1, C1 and D1) and a simultaneous presence of PVP-10 in actual fractions.

To study if this contamination could be quantitatively related to the presence of PA (measured by its absorbance at 530 nm), the concentrations of PA and protein were determined simultaneously in similar experiments, which also included extracts prepared in phosphate buffer with PVP-10 (Fig. 3). The relationship expressed by the straight line (calculated by the least-square method as in Fig. 2) showed a good correlation between the concentrations of proteins and PA, suggesting a stoichiometric relationship between them. However, the simultaneous absence of PA and protein, as indicated by the extrapolated line, was not in complete agreement with the results in Fig. 1, C1 and D1.

Proanthocyanidin contamination of the protein molecules

Proteins and high molecular weight PA may coexist independently in the void fractions or alternatively, they may form soluble protein–PA associations. To determine which these two alternatives actually occurred, the affinity between an insoluble PVP phase (Polyclar AT) and polyphenols was utilized. After an initial extraction in acetate buffer with PVP-10 followed by gel filtration with the same buffer, the absorbance at 280 nm and the protein yield were analysed (Fig. 4, A1 and B1). The three main proteinaceous fractions were then pooled, added to a Polyclar AT column, and eluted with the same buffer. The fractions were analysed as above (Fig. 4, A2 and B2). An evident reduction in absorbance was detected. By comparing these data with those from the control experiment (Fig. 4, A3), the persisting absorbance (Fig. 4, A2) was subsequently attributed to the presence of the soluble PVP-10 originally used in the extraction. Almost all of the protein disappeared (Fig. 4, B2). The simultaneous binding of the protein and the material responsible for the absorbance at 280 nm (cf. Figs 2 and 3), suggested that soluble associations existed between the protein and the PA moieties.

Low temperature during the preparation of the homogenates and during the gel filtrations and the presence of PVP-10, with its postulated affinity for phenolics, could inhibit polyphenol oxidase activity. Consequently, the formation of PA–protein associations might not depend on reactions between proteins and oxidized phenolics. To confirm this, both extractions and gel filtrations were carried out with additions of either dithionite or 2-mercaptoethanol, which in separate tests completely inhibited polyphenol oxidase activity. After gel filtrations, the calculated 280 nm absorbance for the protein moiety was related to the total absorbance at the same wavelength (Table 2). Independent of the enzyme inhibition, there was protein contamination, thus excluding the possibility that the PA–protein associations were caused by polyphenol oxidase activity.

The results from extractions with the detergent Tween 80 (Table 1) suggested that hydrophobic interactions might also exist which could induce protein–PA interactions. To test the properties of the solubilized protein–PA molecules in this respect, adsorption experiments with Phenyl-Sepharose CL-4B (P-S) were conducted.

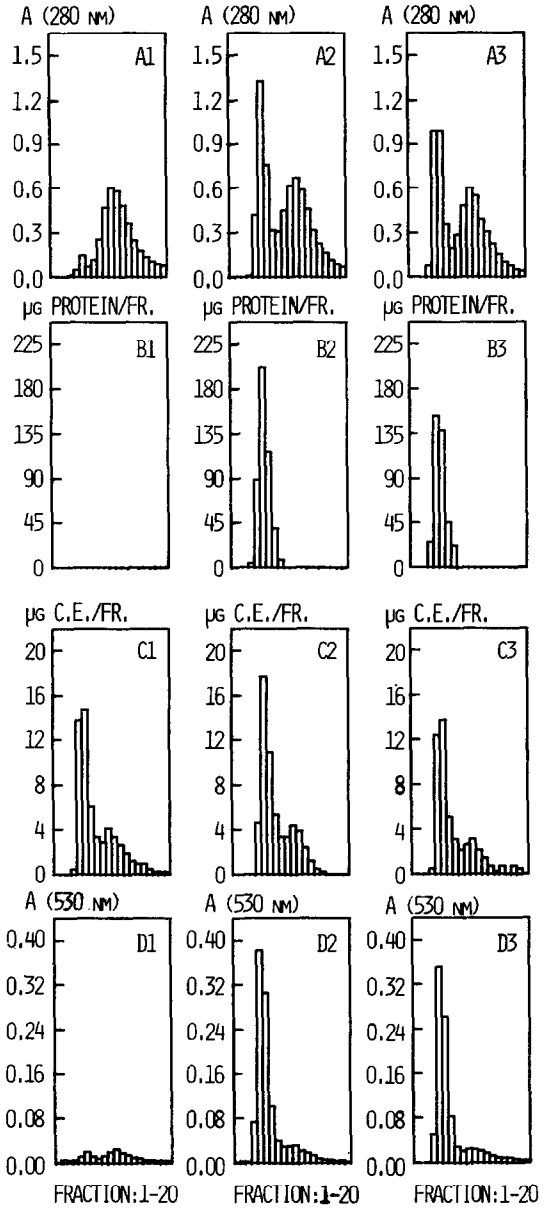

Fig. 1. Separation patterns for 280 nm-absorbing materials (A1-A3), proteins (B1-B3), catechin equivalents (C1-C3), and proanthocyanidins (D1-D3) from gel filtrations of Scots pine needle homogenates; prepared in plain phosphate buffer (A1-D1), buffer with PVP-10 (A2-D2) and buffer with a combination of PVP-10 and 0.5 M NaCl (A3-D3).

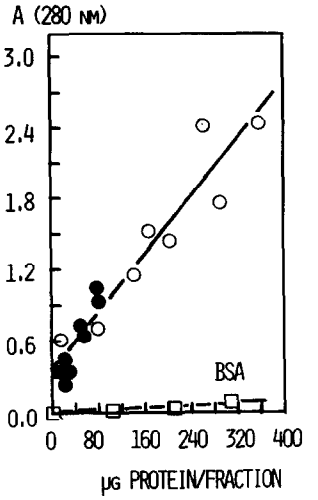

Fig. 2. The relationship between 280 nm absorbance and protein concentration of fractions from gel filtrations of Scots pine needle homogenates prepared with PVP-10 in borate (open circles) and acetate buffers (filled circles). Control with bovine serum albumin ('BSA').

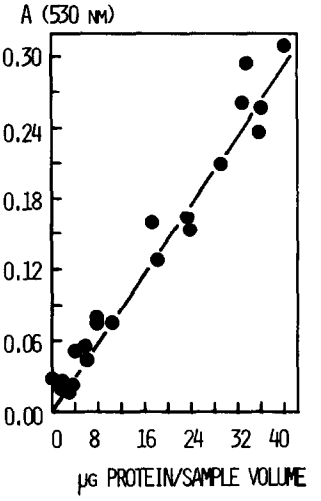

Fig. 3. The relationship between proanthocyanidin and protein concentration in fractions from gel filtrations of Scots pine needle homogenates prepared in acetate, phosphate and borate buffers with PVP-10.

Homogenates were prepared in phosphate buffer with PVP-10. After gel filtrations, the proteinaceous fractions were individually shaken with a slurry of P-S. Adsorption of protein and PA was studied in relation to identical samples without P-S. Positive correlations were observed between adsorption of protein and PA (Fig. 5), which further supported the concept of a persisting 'tanning' of the solubilized protein molecules. The enhanced effects of increased temperature and ionic strength were in accordance with an adsorption by hydrophobic interaction.

DISCUSSION

The occurrence of proanthocyanidins in Scots pine needles [5] and their ability to precipitate proteins [3] suggest that the low protein yields resulting from extractions in acidic or neutral buffers (Table 1) are due to the formation of protein–tannin precipitates. The increase in protein solubility caused by addition of polyvinylpyrrolidone, utilized here as well as in several other studies [1, 2], is generally ascribed to PVP's effects on hydrogen bonds [6, 7]. The high affinity between a proanthocyanidin and soluble PVP has also been shown in competitive precipitation experiments using pure proteins

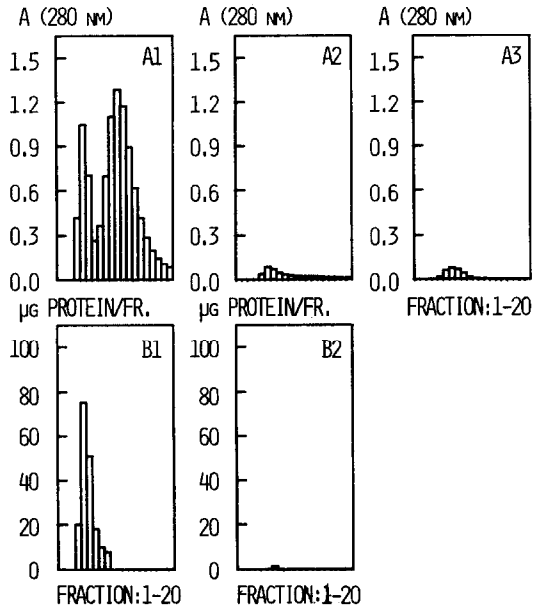

Fig. 4. Simultaneous proanthocyanidin and protein adsorption on a Polyclar AT column (A2 and B2, respectively) after initial separation by gel filtration (fractions no. 4–6, A1 and B1) of Scots pine needle homogenate prepared in acetate buffer with PVP-10. Control with PVP-10 alone (A3).

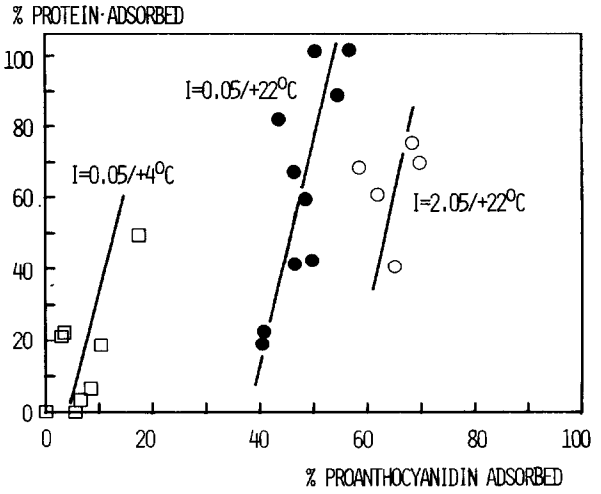

Fig. 5. Effects of temperature and ionic strength on the simultaneous adsorption on Phenyl-Sepharose CL-4B of proanthocyanidin and protein from Scots pine needle homogenates prepared in phosphate buffer with PVP-10.

Table 2. Effects of dithionite and 2-mercaptoethanol on the relative absorbance of protein at 280 nm in fractions from gel filtrations of Scots pine needle homogenates prepared in phosphate buffer with PVP-10

	100 × *A* (280 nm, protein)/*A* (280 nm, total)		
Fraction No.	Control	Dithionite 50 mM	2-Mercaptoethanol 10 mM
4	0.0	7.7	6.9
5	2.6	7.0	4.9
6	3.6	6.6	4.3
7	2.9	4.2	4.0
8	2.5	2.0	0.0
9	4.0	0.2	0.0
10	0.0	0.4	0.0

[8]. The participation of hydrogen bonds in proanthocyanidin–protein interactions in the present needle extracts is also suggested by the positive effects incurred by increasing the ionization of phenolic hydroxyl groups (p*K*s 8.5 or higher [7]) at higher pH levels. The positive effect of the borate, due to its tendency to complex with vicinal hydroxyl groups in the tannins, supports the same concept.

However, detergents which can improve the yield of protein extractions from various plant materials have also been used earlier with Scots pine needles [9, 10]. The general ability of detergents to disrupt hydrophobic interactions [11], their solubilizing effects on tannin–protein precipitates [12], and their ability to release protein from immobilized proanthocyanidins [13] also stress the importance of considering hydrophobic interactions in protein–tannin interactions.

Since the dissolved proteins from Scots pine could be adsorbed both on Polyclar AT by hydrogen bonds (Fig. 4) and on Phenyl-Sepharose gel by hydrophobic interaction at a low ionic strength (Fig. 5)—properties unexpected for pure proteins—the proanthocyanidins should be considered as important links in the absorbing media. Moreover, both types of interactions have been shown for proanthocyanidins in adsorption [13, 14] and precipitation experiments [15].

It might be expected that by solubilizing a protein–tannin complex, all interactions between the moieties would be eliminated. However, the documented existence of soluble complexes [13, 16], the knowledge that only tannin molecules above a minimum size can precipitate proteins [3], and the view that both tannin–protein and polyphenol–polyvinylpyrrolidone interactions are equilibrium processes [6, 16] suggest that soluble tannin–protein associations probably occur more often than generally believed [2]. The present study with Scots pine needles further supports this concept.

Compounds which show both hydrogen bonding and hydrophobic interactions are simultaneously hydrophilic and hydrophobic ('amphiphilic substances' [17]). Their mode of interaction is determined by the nature of the medium in which they occur. A hydrophilic medium will favour hydrophobic interactions; a hydrophobic medium will strengthen hydrogen bonds [13, 18]. Thus, the observed 'tanning' of the solubilized needle proteins would mainly depend on hydrophobic interactions. The efficiencies of both PVP-10 and Tween 80 in the original extractions (Table 1) may also be consistent in this respect. Since hydrogen bonds are basically of an electrostatic nature [18], increased ionic strength would be expected to weaken such bonds. At the same time, hydrophobic interactions would be enhanced [13, 17], possibly explaining why increased ionic strength affects extraction (Fig. 1,

A3-D3) and adsorption with Phenyl-Sepharose gel (Fig. 5) in different ways.

EXPERIMENTAL

Plant material. Needles were collected from the terminal shoots of the lower crown branches of one open-growing Scots pine (*Pinus sylvestris* L.) at Uppsala, Sweden (C-needles). During June–August, when new C-needles were developing, C + 1-needles were used. In comparative expts all needles were taken from one and the same shoot.

Extraction. Two needles (one needle pair, 70–110 mg fr. wt) were fixed in liquid N_2 (−196°) and homogenized with a pestle in a pre-chilled (−20°) porcelain mortar. The first 1.0 ml of extraction medium yielded a mixture of ice and crushed material. The next 1.0 ml produced a half-frozen slurry and the final 3.0 ml yielded an ice-cold suspension which was immediately filtered. Buffers of the same ionic strength (I = 0.05) were used as extraction media: acetate, pH 5.0; phosphate, pH 7.0; glycine, pH 9.0, and borate, pH 9.0 with polyvinylpyrrolidone (PVP-10) and polyoxyethylene sorbitan monooleate (Tween 80), mostly as 1.0% soln (w/v) (exceptions in Table 1).

Filtration was done through a Millipore filter (2.5 cm diam, HA 0.45 μ with a pre-filtrum, AP 20022000). The clear, cell-free homogenate was then kept on ice or directly used in subsequent expts.

Gel filtration was conducted at +4° using a Sephadex G-50/M column (1.6 × 9 cm) equilibrated and run with the same buffer as that used for the actual homogenate but without further additives. The flow rate was set at 2.0 ml/min and each fraction included 2.0 ml. The expts shown in Table 2 were conducted at +22° using a Sephacryl S-200/Superfine column (1.6 × 13.5 cm) with distilled water at a flow rate of 0.5 ml/min and a fraction vol. of 2.0 ml. Dithionite (50 mM) and 2-mercaptoethanol (10 mM) were used here as additives for both the extraction and filtration procedures. Samples (2.0 ml of each homogenate) were injected into a separate container (total vol. 6 ml) connected to the top of the column. The void vol. was determined using Blue Dextran (2.0 ml, 1.0%, w/v). In similar control expts with PVP-10 and Tween 80, both substances appeared in the same fractions as the Blue Dextran. The first four fractions appearing after the void vol. have been termed 'void fractions'. Filtrations were also done through a Polyclar AT column (1.6 × 6 cm, Fig. 4) using the same procedure as those described for the Sephadex-column.

Batch experiments. Hydrophobic interactions were studied with Phenyl-Sepharose CL-4B as a slurry in phosphate buffer (150 mg wet wt/ml and pH 7.0, ionic strength 0.05, Fig. 5). From each void fraction after gel filtration, 0.9 ml was added to 0.5 ml of the Phenyl-Sepharose suspension and shaken for 20 min at +4° and room temp, respectively. Ionic strength was adjusted by adding NaCl. After Millipore filtration, the percentages of adsorbed protein and proanthocyanidin were calculated by comparing the unadsorbed amounts in identical samples with and without Phenyl-Sepharose.

Analyses of total phenolics. The absorbance at 280 nm was used to estimate the amount of phenolics present [cf. 19]. A double-beam spectrophotometer (Beckman DB) was used with appropriate blanks for both these and the colorimetric measurements.

Proanthocyanidin (PA) analyses. PA has been estimated by two independent methods. In the vanillin–HCl method [20], 2.0 ml of a freshly prepared vanillin soln in MeOH (4%, w/v) was mixed with 1.0 ml HCl (37%, w/v) and 0.1 ml of the sample. After 15 min in darkness at room temp, its absorbance at 500 nm was recorded. The results have been presented in terms of μg catechin equivalents/fraction using (+)-catechin as the reference substance. Since the PA concns in the void fractions were of primary interest, no corrections for the occurrence of any low M_r anthocyanidins were made. With respect to flavonoids, the vanillin–HCl test is specific for *meta*-oriented hydroxy groups in the A ring and with a single bond at the 2,3-position in the heterocyclic ring [21].

The characteristic formation of anthocyanidins obtained by heating PA in acidic soln [22] was also used to estimate PA. In this method 0.5 ml of the sample and 1.0 ml HCl (37%, w/v) were held in a test tube (1.5 × 15 cm) immersed in a boiling water-bath under dim light for 15 min. Maximum absorbance was read at 530 nm instead of 550 nm, which is the wavelength used after treatment with dil. HCl in *n*-BuOH [22, 23]. Due to the lack of a suitable reference substance and the conflicting values given for extinction coefficients of isolated PA [19, 24, 25], no attempts were made to calculate the PA content.

Protein analyses. In the expt shown in Table 1, analyses were made by heat-fixation of the samples (1 × 5–5 × 5 μl) on filter paper (Munktell No. OB). After staining with Coomassie Brilliant Blue (C.B.B. R-250, 0.1% w/v in H_2O–*iso*-PrOH–HOAc, 65:25:10), the dried spots were eluted with 5.0 ml, 1.0%, w/v sodium dodecyl sulphate (SDS) in water. The absorbance was measured at 595 nm using a corresponding SDS-soln with a blank paper spot as reference. Calibration was made with cryst. bovine serum albumin (BSA). This method, adapted from Esen [26], was used because the combination of detergent (Tween 80) and C.B.B. gave a blank reaction. In further analyses, 0.1 ml of the sample was added to 3.0 ml of 0.01%, w/v C.B.B. G-250 in EtOH–H_3PO_4–H_2O (1:2:17), [27, 28]. The absorbance was read at 595 nm using the stain soln as a blank. The same BSA as described above was used for calibration. Its absorbance at 280 nm in water (ε = 0.00066 ml/μg) was utilized for calculating expected absorbances for various protein concns (Fig. 2, Table 2).

Enzyme tests. The activity of polyphenol oxidase, (EC 1.10.3.1) at room temp. was measured at 460 nm in 0.1 M phosphate–citrate buffer, pH 6.0 with 4-methylcatechol (12 mM) as substrate. Addition of 2-mercaptoethanol (10 mM) completely inhibited enzyme activity. Expts using (+)-catechin (12 mM) as substrate gave similar results.

Recording. The transmission signal from the photometer was transformed into absorbance data by an external log-converter. It was then digitalized and transferred to a microcomputer connected to a printer and plotter.

Chemicals. Sephadex G-50/M, Sephacryl S-200/Superfine, and Phenyl-Sepharose CL-4B were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Coomassie Brilliant Blue R-250 from Merck, Darmstadt, Germany; Coomassie Brilliant Blue G-250 and Tween 80 from Serva, Heidelberg, Germany; and Polyclar AT from GAF, Stockholm, Sweden. The latter compound was purified according to ref. [6].

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