Polyphenol Oxidase from Sweet Potato: Purification and Properties

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Polyphenol oxidase (PPO, EC 1.14.18.1) extracted from sweet potato root [*Ipomoea batatas* (L.) Lam.] was purified 189-fold by precipitation with ammonium sulfate and elution from columns of Sephadex G-25, DEAE-cellulose, and Sephadex G-100. Polyacrylamide gel electrophoresis of the purified preparation revealed that PPO was highly purified by the procedure adopted. The purified enzyme had an estimated molecular weight of 96 000 and K_m values of 26, 8, 5, and 96 mM for 4-methylcatechol, chlorogenic acid, caffeic acid, and catechol, respectively. The optimum pH varies from about 4.0 to 6.5, depending on the substrate. PPO activity was inhibited by *p*-coumaric and cinnamic acids, sodium metabisulfite, dithioerythritol, ascorbic acid, L-lysine, D-phenylalanine, L-methionine, glycine, L-isoleucine, and L-glutamine. Heat inactivation between 60 and 80 °C was biphasic. Sucrose, $(NH_4)_2SO_4$, NaCl, and KCl appeared to be protective agents of sweet potato PPO against thermal denaturation.

INTRODUCTION

Polyphenol oxidase (PPO; EC 1.14.18.1) catalyzes the oxidation of mono-, di-, and polyhydric phenols to *o*-quinones. Because of the deleterious effect of enzymatic browning on food products, PPO has been extensively studied in a variety of tissues (Vámos-Vigyázó, 1981; Mayer and Harel, 1979; Rolle et al., 1991).

Sweet potato root is a significant contributor to worldwide caloric needs (Walter et al., 1984). This root is susceptible to browning reactions that affect quality and consumer acceptance. Although sweet potatoes do not discolor when cut or sliced, they do darken or brown when peeled and heat-processed, and the tissue damage caused by these processes results in activation of PPO and leads to discoloration of the product (Scott and Kattan, 1957; Arthur and MacLemore, 1956). High PPO activity is generally associated with sweet potatoes which darken during processing (Scott and Kattan, 1957).

In addition, the enzymatic browning tendency of sweet potato cultivars could also be related to phenolic content, especially to the levels of chlorogenic acid and isochlorogenic acid, the most abundant components comprising 80% or more of the total phenolic compounds present in the root (Porter et al., 1976; Walter et al., 1979; Walter and Purcell, 1980). Although the presence of PPO in sweet potato has been clearly demonstrated, little is known about the physicochemical properties of PPO from this source. Hyodo and Uritani (1965) isolated and purified the enzyme and characterized its electrophoretical and immunological properties. To our knowledge, this is the only study related to purification and characterization of sweet potato PPO. Thus, the objective of the present study was to obtain more information on PPO from this source.

MATERIALS AND METHODS

Materials. Cytochrome *c*, soybean trypsin inhibitor, ovalbumin, bovine serum albumin, alkaline phosphatase, DEAE-cellulose, Sephadex G-25 and G-100, L-cysteine, chlorogenic acid, caffeic acid, and phenylenediamine were purchased from Sigma Chemical Co., St. Louis, MO. Catechol and 4-methylcatechol were purchased from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Sucrose, NaCl, KCl, $(NH_4)_2SO_4$, and Na_2SO_4 were of analytical reagent grade. Healthy sweet potato roots [*Ipomoea batatas* (L.) Lam.] cv. Rosada obtained from a local producer were used in the study. The roots were selected, washed, hand-peeled, chopped, and ground in liquid nitrogen.

Methods. *Extraction and Purification of PPO.* Portions (30–50 g) of the frozen tissue powder were homogenized in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.2 mM L-cysteine and Amberlite IRA 938 (2 g/10 g of tissue) in an Ultraturrax homogenizer. The suspension was filtered through four layers of cheesecloth and centrifuged at 25000*g* for 40 min. The supernatant (crude extract) was brought to 30–90% saturation with $(NH_4)_2SO_4$. The precipitate was separated by centrifugation as described above and dissolved in 5 mM sodium phosphate, pH 7.0, containing 0.2 mM L-cysteine. PPO was purified essentially as described by Lourenço et al. (1990) using a combination of gel filtration on Sephadex G-25 and G-100 and elution from DEAE-cellulose.

Measurement of PPO Activity. PPO activity was measured with 4-methylcatechol, chlorogenic acid, caffeic acid, and catechol as substrates at pH 4.0, 4.5, 5.5, and 6.5, respectively, as essentially described by Lourenço et al. (1990).

Protein Determination. Protein concentration was determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard. Absorbance at 280 nm was used to monitor protein in the column eluates.

Molecular Weight. The molecular weight of PPO was determined on a Sephadex G-100 column (2.5 × 60 cm) using cytochrome *c* (12 000), soybean protein inhibitor (21 000), ovalbumin (43 000), bovine serum albumin (68 000), and alkaline phosphatase (100 000) as molecular weight standards according to the method of Whitaker (1963).

Gel Electrophoresis. Electrophoresis was performed on 7% polyacrylamide gel according to the method of Davis (1964). Protein bands were stained with Coomassie brilliant blue R-250. PPO activity was detected by incubating the gels in a reaction mixture containing 2 mM chlorogenic acid and 0.1 M phenylenediamine for 1 h.

Heat Stability. Enzyme solutions (0.10 mg of protein/mL) were heated at different temperatures (60–90 °C). The remaining activity was assayed at appropriate time intervals under standard conditions. To study the effect of additives on heat stability, PPO was incubated in the presence either salt or sugar.

pH Optimum and pH Stability. PPO activity as a function of pH was determined using chlorogenic acid, catechol, 4-methylcatechol, and caffeic acid as substrates in McIlvaine's buffer solutions (pH 3.0–8.0). The pH stability was determined by incubating the enzyme in the above buffer (pH 3.0–7.5) for 2 h at 30 °C. PPO activity was assayed under standard conditions with catechol and chlorogenic acid as substrates.

Inhibitor Effects. To determine the effect of inhibitors, reactions were run at 30 °C in the presence and absence of inhibitors.

All enzyme assays were performed in triplicate.

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Table I. Purification of Polyphenol Oxidase from Sweet Potato Root

procedure	total units	total protein, mg	sp act., units/mg of protein	purifn, factor
crude extract	390 000	468.4	832.6	1
$(NH_4)_2SO_4$ (30–90%)	135 000	161.8	834.3	1
Sephadex G-25	239 000	94.0	2 542.3	3
DEAE-cellulose	25 000	0.16	157 875	189.5
Sephadex G-100	3 080	0.02	158 012	189.7

RESULTS AND DISCUSSION

Extraction and Purification of PPO. The activity and protein extracted varied according to the extraction medium used. The buffer-to-tissue ratio was an important factor in the extraction of the enzyme from sweet potato; when the enzyme was extracted using ratios of 2–6:1, the highest specific activity was obtained at the 4:1 ratio. PPO could be extracted by buffer of low molarity (0.02–0.4 M); the maximum yield was achieved at a concentration of 0.1 M. The optimal pH conditions for PPO extraction were also investigated using McIlvaine's buffer and phosphate buffer in the pH range 4.5–7.5. With both buffer systems, higher specific activity yields as a function of pH were obtained in the pH range 5.0–7.0. Sweet potato PPO precipitated over a wide range of ammonium sulfate concentration (0–30, 30–50, 50–70, and 70–90% saturation). Since the highest activities occurred in a broad saturation range (30–90%), this was used as the source of enzyme for purification. The procedure adopted to purify PPO from sweet potatoes is summarized in Table I. The results presented are representative of five separate purification trials, and the elution patterns were highly reproducible. Passage of the 30–90% ammonium sulfate fraction through a column of Sephadex G-25 removed L-cysteine present in the preparation and produced a protein fraction containing 61% of the activity detected in the crude extract with a 3-fold purification. Further purification of PPO was achieved by chromatography on DEAE-cellulose. All PPO activity was eluted in a single peak. The tube peak fraction accounted for at least 10% of the total activity applied to the column. When this preparation was subjected to gel filtration on Sephadex G-100, one sharp peak of activity was observed. Since the fractions eluted from DEAE-cellulose contained more protein per milliliter, higher activity, and a similar purification factor compared to the fractions eluted from Sephadex G-100, they were pooled and precipitated by ammonium sulfate at 90% saturation, dialyzed, and used for the assays. The extent of enzyme purification achieved in this study, 189-fold, is higher than that achieved for the enzyme from a number of other sources (Valero et al., 1988; Park and Luh, 1985; Owusu-Ansah, 1989). The effectiveness of the purification procedure described here was assessed by polyacrylamide gel electrophoresis. The enzyme preparation eluted from the DEAE-cellulose column was free from contaminating proteins and was resolved into two protein bands (a, b) whose position on the gel corresponded to one large activity band observed by specific staining. A similar electrophoretic pattern was observed with the enzyme eluted from Sephadex G-100.

Physicochemical Properties of PPO. The optimum pH values for activity were 4.5, 4.0, 5.5, and 6.5 with chlorogenic acid, 4-methylcatechol, caffeic acid, and catechol as the substrates, respectively. The PPO activity with chlorogenic acid, the most abundant phenolic substrate present in sweet potatoes, reached the highest level compared with the other substrates. The pH stability study revealed that preincubation of the enzyme at pH 4.0–7.5 apparently had no effect on the activity measured at pH 4.5 and 6.5 with chlorogenic acid and catechol as substrates. The molecular weight of sweet potato PPO was 96 000 as estimated by gel filtration on a calibrated Sephadex G-100 column. Although multiplicity of MWs appears to be a common property of numerous PPOs from various sources, the sweet potato enzyme seems to exist in only one molecular form. The subunit MW of PPO determined by SDS–PAGE was 25 000 ± 2000 (Weber and Osborn, 1969), indicating that sweet potato PPO is a tetramer composed of four subunits of similar molecular weight.

Table II. Apparent V_{max} and K_m of Sweet Potato PPO for Four Phenolic Substrates

substrate	V_{max}, % found for chlorogenic acid	K_m, mM
chlorogenic acid	100	8
caffeic acid	82	5
4-methylcatechol	58	26
catechol	49	96

Table III. Effect of Some Compounds on Sweet Potato PPO (Percent of Inhibition)

compd	concn, mM	substrate: chlorogenic acid	caffeic acid	4-methylcatechol	catechol
cinnamic acid	1	53	38	33	0.5
p-coumaric acid	0.3	66	40	40	0
protocatechuic acid	1	28	0	0	0
KCN	1.5	60	0	9	84
thiourea	0.5	80	49	23	68
sodium metabisulfite	0.5	43	30	44	22
	0.7	67	40	69	54
ascorbic acid	0.5	2	9	10	11
	0.7	3	19	30	14
dithioerythritol	0.5	30	57	31	100
	0.7	76	84	62	100
sodium fluoride	5	85	80	93	0

The K_m values of the enzyme for caffeic acid, chlorogenic acid, 4-methylcatechol, and catechol determined according to the method of Lineweaver–Burk (1934) were found to be 5, 8, 26, and 96 mM, respectively (Table II). With catechol as the substrate, the enzyme exhibited classical Michaelis kinetics in the concentration range 0.5–200 mM; beyond this concentration, catechol was found to inhibit the enzyme. Using caffeic acid and chlorogenic acid as substrates, Hyodo and Uritani (1965) reported K_m values of 2.9 and 3.1 mM, respectively, for PPO purified from sweet potato variety Norin 1. Also, the K_m values reported here are higher than those previously reported for PPO from other sources: apples (Janavitz-Klapp et al., 1990), airen grapes (Valero et al., 1988), and apricots (Dijkstra and Walker, 1991). The effect of inhibitors on the enzyme activity with catechol, chlorogenic acid, caffeic acid, and 4-methylcatechol as substrates is shown in Table III. With catechol as the substrate at pH 6.5, cinnamic acid, *p*-coumaric acid, and sodium fluoride had no measurable effect on PPO activity, suggesting that inhibition of sweet potato PPO by these compounds was strongly influenced by pH. The inhibition by *p*-coumaric acid and cinnamic acid with caffeic acid and 4-methylcatechol was of competitive type; K_i values of 1.6 and 0.37 mM calculated from Dixon plots (Dixon, 1953) were found for *p*-coumaric acid, respectively, while K_i values for cinnamic acid were 2.0 and 2.4 mM, respectively. Potassium cyanide strongly inhibited the enzyme with catechol as substrate.

The thiol reagents dithioerythritol (DTE) and sodium metabisulfite at the concentrations indicated were effective inhibitors of sweet potato PPO, while ascorbic acid showed

a relatively poor inhibitory effect. In the assay of PPO with these inhibitors, a lag period was observed before any changes in absorbance were measured, which increased with inhibitor concentration. These inhibitors also delay the start of the reaction catalyzed by mushroom PPO (Golan-Goldhirsh and Whitaker, 1984). These authors also reported that the strongest inhibition of mushroom PPO was observed with dithioerythritol followed by sodium metabisulfite and ascorbic acid. In contrast to our results, Hsu et al. (1988) found that 0.5 mM ascorbic acid completely suppressed the oxidation of Dopa by mushroom PPO. The lack of color development observed in the presence of the inhibitors is due to their ability to reduce quinones formed by enzymatic oxidative reaction back to colorless *o*-dihydroxyphenols. In addition, complexing of quinones with bisulfite and addition reactions between quinones and thiols can also lead to colorless products (Golan-Goldhirsh and Whitaker, 1984; Vámos-Vigyázó, 1981). A number of amino acids, L-lysine, D-phenylalanine, L-methionine, glycine, L-isoleucine, and L-glutamine, in a wide range of concentrations (10–120 mM) were assayed as possible inhibitors of sweet potato PPO. Regardless of the substrate used, catechol, 4-methylcatechol, or chlorogenic acid, no inhibitory effect was observed at pH 4.0 and 4.5. The only exception was observed with L-lysine, which was able to decrease the enzyme activity by 16 and 39%, respectively, at 100 mM concentration. In contrast to this result, all of the amino acids assayed were effective inhibitors of PPO activity in the oxidation of catechol at pH 6.5. Among the amino acids chosen, L-lysine and D-phenylalanine were the most effective inhibitors (data not shown). The most recent evidence that PPO is inhibited by proteins, peptides, and amino acids was obtained by Kahn (1985) for the enzyme from mushroom, avocado, and banana. This author reported that L-phenylalanine, L-histidine, glycine, and L-lysine in decreasing order of effectiveness inhibited the Dopa oxidation by mushroom tyrosinase. Proteins and amino acids can affect PPO activity by reacting with *o*-quinone products of PPO activity and by chelating the essential copper at the active site of PPO. Mason and Peterson (1965) reported that N-terminal primary amino groups, aliphatic amino groups, and thiol-containing amino acids react with *o*-quinones to give red or brown products. The primary covalent reactions of enzymically generated chlorogenoquinone and caffeoquinone with methionine and lysine are likely to involve the thioether group of methionine and the ϵ amino group of lysine (Pierpoint, 1969a,b; Synge, 1975). In addition, certain amino acids are able to form stable complexes with Cu^{2+} at the active site of PPO and hence make the enzyme ineffective (Khan, 1985; Mathew and Parpia, 1971).

It is well established that some chemical agents stabilize proteins in solution (Crowe et al., 1987). Investigations of the protective action of chemical agents on heat denaturation have been carried out with a number of proteins from different sources. Although the sugars, salts, and polyhydric alcohols have been used as stabilizing agents for the maintenance of biological activity of macromolecules, there are few reports on the effect of these agents on heat inactivation of plant enzymes, particularly of multisubunit proteins. It seemed of interest, therefore, to investigate the possible stabilizing effect of sucrose and salts on the heat inactivation of the purified PPO from sweet potato. The kinetics of the heat inactivation of the enzyme at 60–90 °C are shown in Figure 1. It can be seen that the heat inactivation of PPO between 60 and 80 °C followed a biphasic process. The enzyme activity determined with catechol as the substrate at pH 6.5 was reasonably stable at 60 °C; a gradual decline in activity was observed, with a drop to a value of 80% of the original level after 15 min of heating. As expected, an increase in the heating temperature inactivated PPO more quickly; at 80 °C the loss of activity was rapid, decreasing to 36% of the original value after 2 min of heating. As shown in Figure 1, heating at 90 °C for 1 min fully inactivated PPO activity. Thus, it appears that the heat inactivation of the enzyme is temperature-dependent. When the heated enzyme was assayed with 4-methylcatechol at pH 4.0 as the substrate, similar inactivation curves were obtained (data not shown).

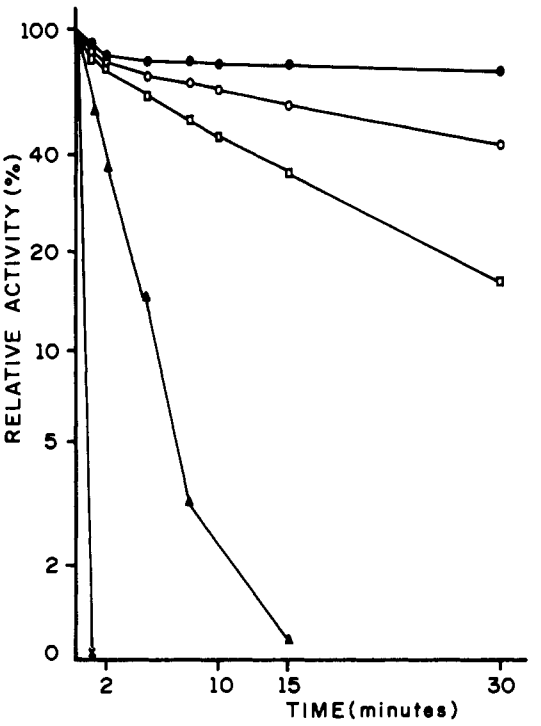

Figure 1. Heat inactivation of PPO at different temperatures. Enzyme solutions containing 0.10 mg of protein/mL in 5 mM sodium phosphate buffer, pH 7.0, were incubated at the indicated temperatures. The remaining activity was determined with catechol as substrate. (×) 90 °C; (▲) 80 °C; (□) 75 °C; (○) 70 °C; (●) 60 °C.

Figure 2 shows the effect of sucrose at 20 and 40% (w/w) on thermal inactivation of PPO at 80 °C. The data shown in Figure 2 reveal that activity was more stable when PPO was heated in the presence of sucrose. In this case, PPO retained 62% of the original activity after 2 min of heating at 80 °C compared to a 36% value obtained in the absence of sugar. In contrast, the enzyme heated in 20% sucrose solution was not markedly protected by sucrose. The heat treatment of the enzyme at 70 °C in 20 and 40% sucrose solutions did not protect the enzyme against heat inactivation as compared to a control heated under similar conditions in the absence of sugar (data not shown). On the basis of the determination of residual activity, Chang et al. (1988) reported that horseradish peroxidase (HRP) is less susceptible to thermal inactivation when heated in 40% sucrose solution. In contrast to the results presented here, these investigators showed that a 20% sucrose solution reduced the thermal stability of HRP. The discrepancies in the behavior of the enzymes upon heating in 20% sucrose solution should be interpreted on the basis of the suggestion of Yoovidhya et al. (1986) that for different enzymes the effect of the same additive can be contrary. According to these authors, this may be due to the nature of the enzyme and particularly to its

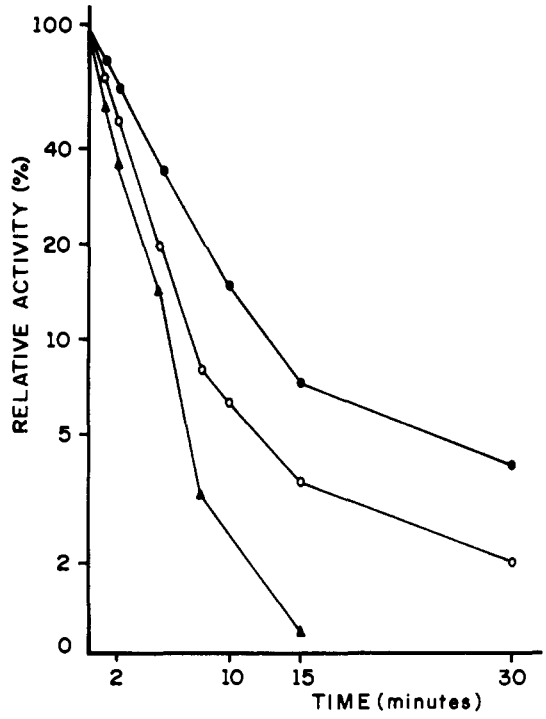

Figure 2. Heat inactivation of PPO in the presence of sucrose. The enzyme solutions containing 0.10 mg of protein/mL were incubated at 80 °C with sucrose. (▲) Control; (●) 40% sucrose concentration (w/w %); (○) 20% sucrose concentration (w/w %). At each time interval the solution was rapidly cooled in an ice/water bath and the remaining activity was assayed with catechol as substrate at 30 °C.

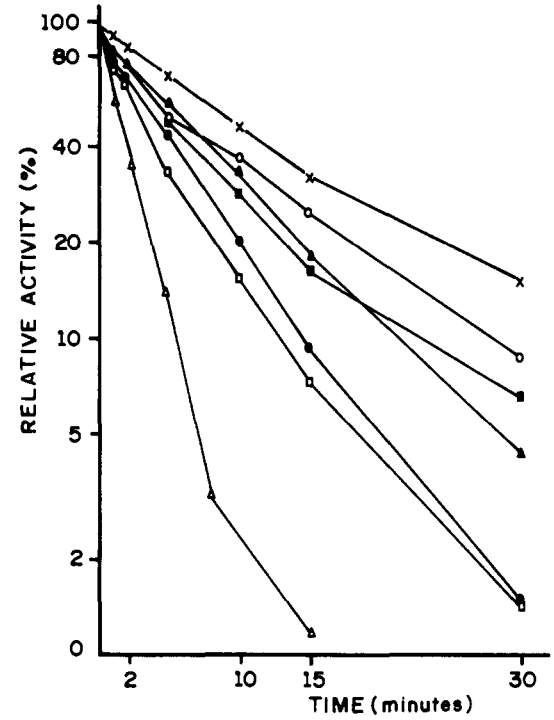

Figure 3. Heat inactivation of PPO in the presence of salts. The enzyme solutions containing 0.10 mg of protein/mL were incubated at 80 °C with salt solutions as indicated. (△) Control; (×) 1 M $(NH_4)_2SO_4$; (○) 1 M Na_2SO_4; (●) 1 M NaCl; (□) 1 M KCl; (▲) 2 M NaCl; (■) 2 M KCl. At each interval the solution was rapidly cooled in an ice/water bath and the enzyme was assayed for remaining activity with catechol as substrate at pH 6.5 and 30 °C.

hydrophilic–hydrophobic balance. The mechanism of heat stabilization of proteins by sugars has been studied by several authors. Back et al. (1979), on the basis of their results, hypothesized that the dominant mechanism by which sugars and polyols stabilize proteins to heat denaturation is through their effect on the structure of water. Along the same line of thought, other researchers have suggested that in sucrose systems the cohesive force of sucrose responsible for the increase in the surface tension of water is a very important factor governing the preferential interaction of proteins with solvent components in aqueous sugar solution and hence the stabilization of proteins against heat denaturation (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a).

The effect of salts on heat inactivation of PPO is shown in Figure 3. The heat inactivation in the presence of salts seemed to induce some heat stabilization of the PPO structure, as measured by loss of initial activity. As shown in Figure 3, the protective effect on PPO activity depends on the concentration and the nature of the salts. Among the salts investigated, a series ranking from most to least effective was as follows: $(NH_4)_2SO_4 > Na_2SO_4 >$ NaCl, KCl. It was also observed that at identical concentrations Na_2SO_4 was more effective than NaCl in protecting the enzyme activity. It has been demonstrated that at high identical concentrations of Na_2SO_4 and NaCl and at a pH value where the protein is stable, the exclusion of the salt from the protein is much stronger for SO_4^{2-}, leading to a preferential interaction of protein with solvent components and protein stabilization (Arakawa and Timasheff, 1982b). The effect of salts on protein stability depends on the concentration and/or ionic strength of the salt. At low salt concentration ($\mu < 0.5$) the stabilizing effect of electrolytes on protein conformation has been attributed to an electrostatic response (von Hippel and Schleich, 1969). At $\mu > 0.5$ the ability of salts to stabilize protein structures has been related to the preferential hydration of the protein molecule as a result of a salt-induced alteration of the water structure in the vicinity of the protein and can be referred to as a lyotropic effect (Arakawa and Timasheff, 1982b).

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Registry No. PPO, 9002-10-2; 4-methylcatechol, 452-86-8; chlorogenic acid, 327-97-9; caffeic acid, 331-39-5; catechol, 120-80-9; cinnamic acid, 621-82-9; *p*-coumaric acid, 7400-08-0.