In Vitro Protein Polymerization by Quinones or Free Radicals Generated by Plant or Fungal Oxidative Enzymes

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ABSTRACT

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A model system of horseradish peroxidase, mushroom polyphenol oxidase, or sodium periodate and a phenolic compound was found to polymerize and precipitate soluble proteins. Polyacrylamide SDS gel electrophoresis revealed the presence of protein dimers, trimers, and higher polymers that formed by protein reaction with quinones or free radicals. Physiological concentrations of widespread naturally occurring phenolics, hydroxycoumarins, and flavonoids were capable of participating in the crosslinking reaction. The conditions under which the crosslinking reaction occurred were studied, and its possible mechanism and biological significance is discussed.

Additional key words: peroxidase, polyphenol oxidase, tyrosinase, chlorogenic acid, phenolics.

Oxidative enzymes including peroxidase (23), polyphenol oxidase (17), and laccase (11) occur widely in plants, fungi, and animals. Peroxidase catalyzes the oxidation of reduced hydrogen donors such as phenolic compounds in the presence of the oxidant, hydrogen peroxide. Polyphenol oxidase and laccase catalyze the oxidation of phenolics in the presence of oxygen. Plants and fungi contain a large number of phenolics (10) including phenols, hydroxycoumarins, and flavonoids which may serve as substrates. Once a phenolic substrate is oxidized, the quinone or radicals formed may mediate oxidation of other phenolics which are not a substrate for a given enzyme. Thus, indirectly many phenolics and other compounds which are not substrates can be oxidized to quinones by these enzymes.

Quinones are directly involved in plant, fungal, and animal pigmentation, insect cuticle formation, and in plant secondary cell wall formation (10,17). They are implicated as being important in fungal sporophore cell wall formation (4), and in both plant (9,22,30) and fungal (2,13) disease resistance including defense against insect and nematode attack (12,28). Oxidation of phenolics is closely correlated with, and perhaps essential to, the development of sexual structures in fungi (8,14) and slime molds (7) and has been reported to be involved in the regulation of plant growth (29) and fungal metabolic processes (3,5,16).

Often the mechanism of these processes may be explained by the ability of quinones or free radicals formed by the oxidase enzymes to react or condense with other phenols or nucleophilic chemical groups such as amino or sulfhydryl groups (18,21) in protein. In this way phenolic compounds may react with biologically important chemical compounds, inactivate enzymes; or agglutinate or crosslink proteins, nucleic acids, and even whole cells.

Based on earlier studies with an oxidase system (27) we report additional data with model systems using horseradish peroxidase (E.C. 1.11 . 1.7) and fungal polyphenol oxidase (E.C. 1.14 . 18.1) which crosslink proteins at low concentrations of the oxidative enzymes and phenolic substrates. Changing the concentration or type of: oxidative enzyme (or using periodate), phenolic compound (including several classes of natural compounds), or the protein to be crosslinked was found to affect the extent and/or the rate of crosslinking. The conditions necessary for crosslinking and the stability of the crosslinked protein were investigated.

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MATERIALS AND METHODS

Formation of polymeric products. The following proteins were utilized: ribonuclease (Calbiochem, bovine pancreatic, A grade, five times crystallized, RNase), cytochrome c (Calbiochem, equine heart, grade A), lysozyme (Sigma, egg white, grade I, three times crystallized), bovine serum albumin (Pentex crystallized), peroxidase (HRP, Sigma, horseradish, Type II, 152 units per milligram purpurogallin assay, RZ = 1.5), polyphenol oxidase (Worthington, *A. bisporus,* 700 units per milligram tyrosine assay), glucose oxidase (E.C. 1.1 . 3.4, Worthington, *A. niger*, 121 units per milligram), catalase (E.C. 1.11 . 1.6, Worthington, bovine liver, lyophilized). Hydrogen perioxide (H_2O_2–30%) and all other chemicals were analytical reagent grades from commercial sources.

Three oxidative systems were used: peroxidase (20 μg/ml HRP, 55 units per milliliter, expressed as μmoles of pyrogallol oxidized per minute) with various concentrations of H_2O_2; polyphenol oxidase (1.5 μg/ml, 790 units per milliliter, expressed as μmoles of tyrosine oxidized per minute) in the presence of air as the source of oxygen; or periodate (2.4 mM sodium periodate). A typical reaction mixture consisted of: a protein to be crosslinked, an oxidative system, a phenolic compound as the crosslinking agent, and a buffer. Each component was dissolved separately in 10 mM sodium phosphate buffer, pH 6.8, with an appropriate volume of each solution pipetted into a test tube. The H_2O_2, polyphenol oxidase, or periodate were added last to initiate the reaction, the test tube then loosely covered with parafilm; and after incubation at room temperature for a given time period, the reaction was terminated by addition of the denaturing solution for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Unless otherwise stated, all reactions were carried out for 20 hr.

A standard reaction mixture containing 2 mg/ml (0.15 mM RNase, 20 μg/ml HRP, 2.4 mM H_2O_2, 0.6 mM chlorogenic acid, which was allowed to react for 20 hr, was used as the standard to which other treatments were compared. Except where alternate procedures are given, all other reaction mixtures were prepared identically to the standard reaction mixture.

The extent of the crosslinkage from various reaction mixtures was judged by comparison of the number and density of new oligomeric protein bands on SDS polyacrylamide gels of a given reaction mixture against both a control with no oxidative system treated in a similar manner and a standard reaction mixture with chlorogenic acid.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The reaction mixtures were incubated with 1% (w/v) 2-mercaptoethanol, 4 M urea, 1% SDS (Bio-Rad, Electrophoresis Purity Grade), and 15% (w/v) sucrose at 45 C for 45 min. Bromophenol blue dye (0.1 ml saturated solution in H_2O) was added to the samples after the incubation. The gel contained per liter: 7.5 g acrylamide, 0.93 g *bis*-acrylamide, 2.0 g SDS, 1.0 ml N,N,N′,N′-tetramethylethylenediamine, 0.1 M sodium phosphate buffer, pH 6.8, and 75 μg ammonium persulfate to initiate polymerization of the gel in 5 mm I.D. × 10 cm glass tubes. Gels were pre-electrophoresed for 5–10 min, then an appropriate volume of the reaction mixture containing 40 μg of denatured protein was applied to the top of the gel by syringe. A current of 15 mA per tube was then applied until the bromophenol blue band had migrated 5 cm. Gels were fixed in 7.5% (v/v) acetic acid; stained 3 hr in 0.02% Coomassie brilliant blue R250, 7.5% acetic acid; destained 12 hr in 15% methanol (v/v), 7.5% acetic acid; and stored in 7.5% acetic acid. Photographs of gels were taken with Kodak plus-X (ASA 125) black and white film and a red filter.

RESULTS AND DISCUSSION

Crosslinking of proteins by oxidative systems. Densitometric tracing of an SDS polyacrylamide gel of a standard reaction mixture with a Joyce Chromascan (Joyce - Loebl & Co., LTD, England) with a red filter indicated new oligomeric protein bands from the dimer through the pentamer (Fig. 1). In the absence of added RNase, although the reaction mixture browned, no new bands were detected. Plotting the logarithm of the molecular weight of each respective protein oligomer band against its migration gave a straight line consistent with the conclusion that the bands were protein oligomers (Fig. 2). Similar results were obtained when using a polyphenol oxidase or a periodate oxidative system.

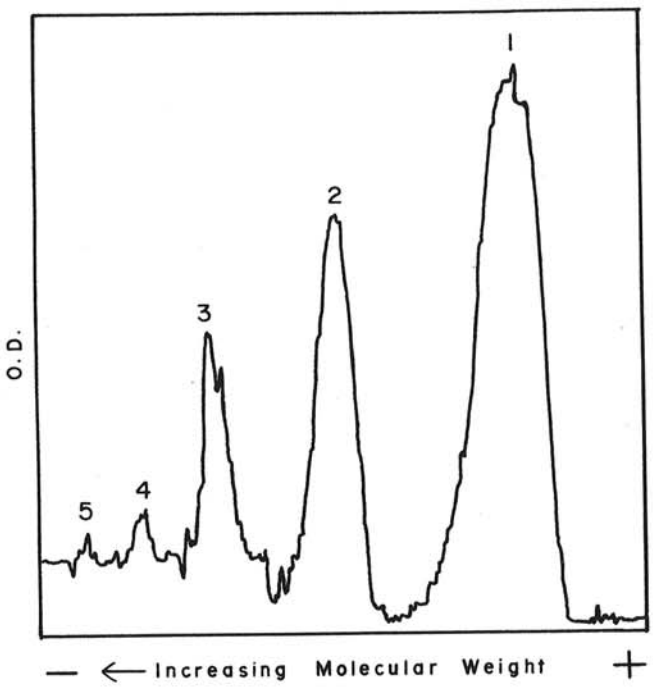


Fig. 1. Densiometric trace of an SDS polyacrylamide gel containing polymerized ribonuclease. The gel was loaded with 40 μg of ribonuclease from a reaction mixture of 2 mg/ml ribonuclease polymerized with a horseradish peroxidase (20 μg/ml)–hydrogen peroxide (2.4 mM) oxidative system with chlorogenic acid (0.6 mM) as the crosslinking agent in 10 mM pH 6.8 sodium phosphate buffer. The reaction was carried out for 20 hr at room temperature (standard conditions) and after electrophoresis the gels were stained with Coomassie brilliant blue R250. New oligomeric bands from dimer through the pentamer (peaks 2 through 5) were present following the oxidation of the chlorogenic acid. Only the monomer (peak 1) was clearly seen in the absence of peroxidase or hydrogen peroxide.

The effect of the peroxide concentration. With the peroxidase oxidative system and a range of H_2O_2 concentrations from 0.075 mM to 9.6 mM, the maximum crosslinkage occurred between 1.2 mM and 2.4 mM H_2O_2 (Fig. 3A, gels 6 and 7). Thus, the optimum ratio of H_2O_2 to chlorogenic acid was between 2:1 and 4:1. A small amount of dimer formed in the absence of chlorogenic acid presumably due to free radical reactions initiated by H_2O_2, and also in the absence of HRP, possibly due to radical reactions or direct oxidation of the chlorogenic acid. However, optimal crosslinkage was dependent on the presence of HRP, chlorogenic acid, and the proper concentration of peroxide (Fig. 3A). The higher concentrations of peroxide also caused a color shift of the reaction mixture from brown to red.

Polyphenols are reported to be degraded in the presence of H_2O_2 and peroxidase (6), so it was of interest to find if the higher levels of H_2O_2 were able to degrade the protein oligomers which would be suggestive of a polyphenol crosslinkage. To test this, a standard reaction mixture (2.4 mM H_2O_2) was allowed to react at room temperature for 20 hr, then with additional H_2O_2 (9.6 mM) and peroxidase (20 μg/ml) for another 4 hr, and a second time for another 16 hr. Although the reaction mixture was bleached by this treatment, no change in the extent of crosslinkage occurred. An excess of H_2O_2 probably affected the ability of a crosslink to form rather than the stability of a crosslink.

The effect of the ratio of oxidized phenolics to protein. With a peroxidase oxidative system and the concentration ratio of H_2O_2 to chlorogenic acid set at 4:1 (the standard reaction mixture), the concentration of both H_2O_2 and chlorogenic acid were varied together at a fixed ratio. In a range of 0.125 times to 32 times the standard concentration, an optimum was found between 0.5 and 4 times the standard (Fig. 3B, gels 4–6). At concentrations of ×4 and higher than the standard, progressively more protein precipitate formed in the reaction mixture. At the highest concentration, precipitation of the protein occurred immediately upon oxidation of the chlorogenic acid. All precipitates formed were found to dissolve immediately upon addition of the denaturing solution for SDS polyacrylamide gel electrophoresis. Precipitation of the protein in a reaction mixture apparently competed against the crosslinking reaction by removing the protein from solution. Thus,

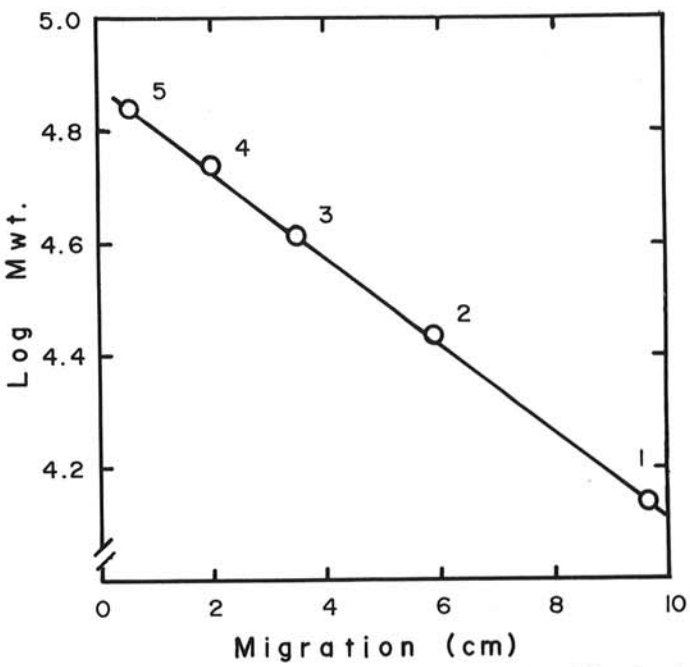
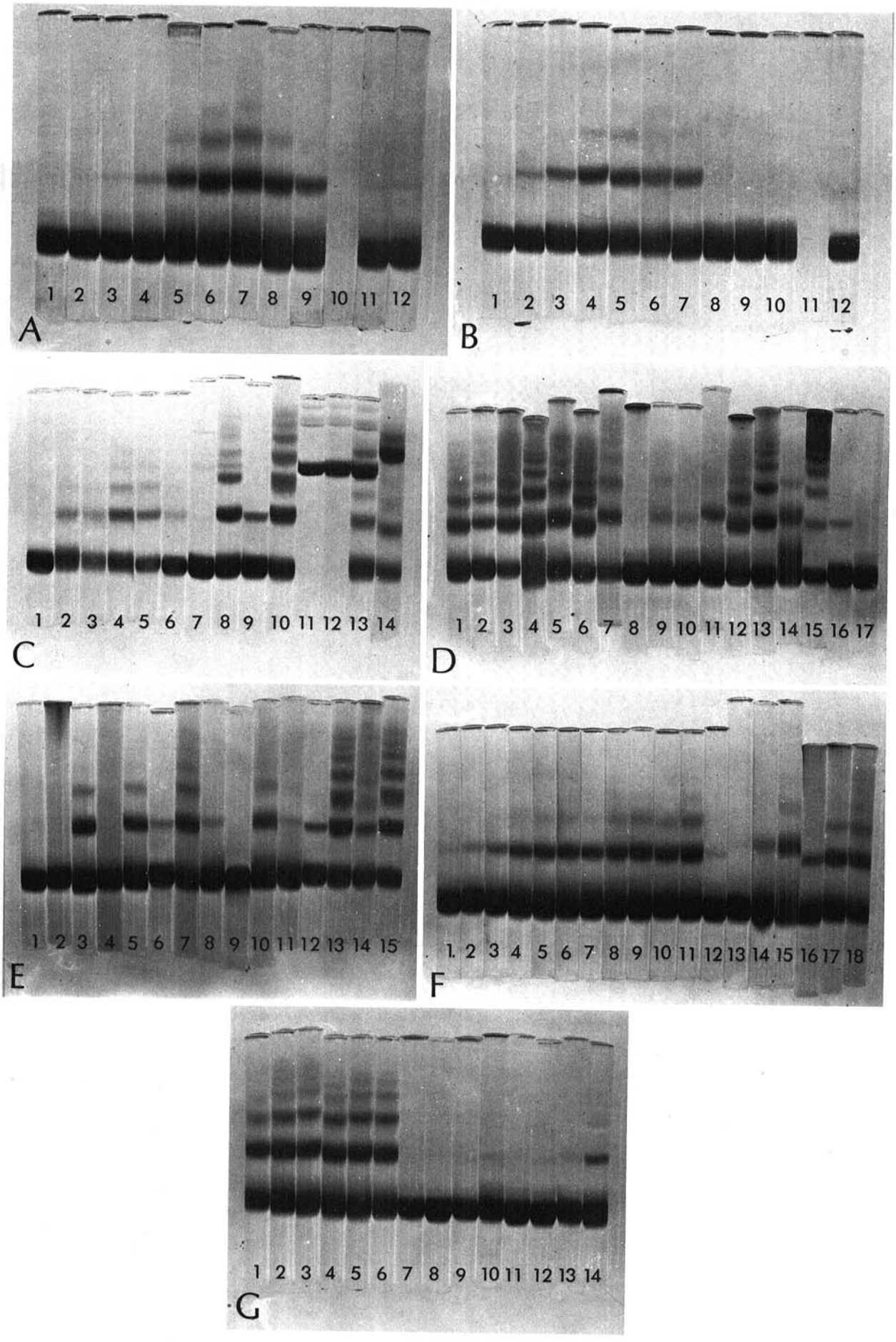


Fig. 2. A plot of the logarithm of the theoretical molecular weight of each polymeric ribonuclease band versus its observed migration. A straight line was obtained consistent with the protein bands being oligomers of ribonuclease for the monomer (peak 1) through pentamer (peak 5) with the densiometric trace from Fig. 1. The equation used to estimate the ribonuclease oligomer molecular weights was: molecular weight = 13,700 ×(oligomer number).


1 2 3 4 5 6 7 8 9 10 11 12
A
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
D
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
E
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
F
1 2 3 4 5 6 7 8 9 10 11 12 13 14
G

quinones were less able to react with the protein precipitate and instead reacted with other quinones to form polyphenols.

Some loss of lysine and sulfur amino acids was found by amino acid analysis of acid hydrolyzates of the polymerized protein. Two unkown ninhydrin-positive peaks were found between the aromatic amino acids and lysine. These were found only when the protein was crosslinked at the optimum concentration of chlorogenic acid and H_2O_2, but not at 16 times this where crosslinkage did not occur. One of the new ninhydrin-positive peaks only occurred after sodium borohydride reduction of the reaction mixture before acid hydrolysis. These new ninhydrin-positive peaks may represent altered amino acids involved in the covalent crosslink.

The effect of the concentration and type of protein on crosslinking. In the standard reaction mixture, maximum crosslinkage occurred with RNase at 2 mg/ml (Fig. 3C, gel 4). Concentrations of 0.5 mg/ml and lower, and 8 mg/ml and higher, gave less crosslinkage. Both lysozyme and cytochrome c at 2 mg/ml crosslinked well under the standard reaction mixture conditions with lysozyme precipitating slightly (Fig. 3C, gels 8 and 10). Bovine serum albumin alone did not crosslink well (Fig. 3C, gel 12); however, a mixture of 1 mg/ml of both bovine serum albumin and ribonuclease or cytochrome c crosslinked with formation of a band in the position expected for the heterodimer (Fig. 3C, gels 13 and 14; Figs. 4 and 5).

Comparison of various phenolic substrates. A large number of phenolics selected from phenols, tannins, hydroxycoumarins, flavonoids, aromatic amino acids, and their derivatives were studied under the standard reaction conditions at 0.6 mM in the place of chlorogenic acid, with peroxidase and H_2O_2 or polyphenol oxidase as the oxidative systems. Under these conditions no phenolic was capable of crosslinking the test protein that was not first oxidized by a given oxidative system. Oxidation here is defined as the ability of an oxidative system to cause a darkening of color in the reaction mixture.

Many, but not all, of the phenolics oxidized were able to crosslink protein (Fig. 3D). The extent of protein crosslinkage and precipitation varied with the phenolic used. Benzidine, a potent carcinogen, was the best crosslinking agent tested (Fig. 3D, gel 15). Tannic acid, present in high concentration in oak tree galls, was the best precipitating agent (Fig. 3D, gel 11). Although tannic acid precipitated some protein without being oxidized, its ability to precipitate protein greatly increased upon its oxidation. When the logarithm of the expected oligomer molecular weight was plotted against band migration from data obtained from densiometric traces of SDS polyacrylamide gels, linear plots were also obtained with protocatechuic acid (Fig. 6A) and naringen (Fig. 6B) as the phenolic crosslinking agent.

Components and reaction conditions necessary for crosslinking. Addition of 2.4 mM L-ascorbate inhibited the crosslinking of RNase under standard reaction mixture conditions (Fig. 3E, gel 4). When L-ascorbate was used at 0.6 mM to replace the chlorogenic acid as hydrogen donor, again, there was no crosslinkage (Fig. 3E, gel 2). Thus: L-ascorbate inhibits the crosslinking reaction, probably by reduction of quinones; and the ability of peroxidase to reduce H_2O_2 alone did not facilitate crosslinking in this model system, but rather a quinone facilitated crosslinking.

Chemical oxidation of the chlorogenic acid by 2.4 mM sodium periodate effectively replaced the peroxidase and H_2O_2 as the oxidative system (Fig. 3E, gel 7). Even though periodate is a stronger oxidant than the expected chlorogenic acid-derived quinone, and both are capable of radical reactions, the quinone was far superior in its ability to crosslink protein since only a dimer was formed in the presence of periodate without addition of chlorogenic acid (Fig. 3E, gel 6). This would be explained if the quinone was required to participate directly in the crosslinking reaction.

Hydrogen peroxide formed from an enzyme system (2.4 mM D-glucose in the presence of 1 unit of glucose oxidase per milliliter) successfully substituted for direct addition of H_2O_2 with comparable crosslinkage (Fig. 3E, gel 10). When a catalase from bovine heart was added in a 15-fold excess (300 units per milliliter) to HRP, it inhibited crosslinking with the glucose oxidase system more than with direct H_2O_2 addition (Fig. 3E, gels 5 and 11).

Fig. 3. SDS polyacrylamide gels. **A,** Effect of the hydrogen peroxide concentration. The reaction mixtures contained 2 mg/ml ribonuclease, 20 μg/ml horseradish peroxidase, 0.6 mM chlorogenic acid, 10 mM sodium phosphate buffer, pH 6.8, and: **gel 1**, no hydrogen peroxide H_2O_2; **2**, 0.075 mM H_2O_2; **3**, 0.15 mM H_2O_2; **4**, 0.30 mM H_2O_2; **5**, 0.60 mM H_2O_2; **6**, 1.2 mM H_2O_2; **7**, 2.4 mM H_2O_2; **8**, 4.8 mM H_2O_2; **9**, 9.6 mM H_2O_2; **10**, 1.2 mM H_2O_2 minus ribonuclease; **11**, 1.2 mM H_2O_2 minus chlorogenic acid; **12**, 1.2 mM H_2O_2 minus horseradish peroxidase. **B,** The effect of the ratio of oxidized phenolic compound to protein. Reaction mixtures contained 2 mg/ml ribonuclease, 20 μg/ml horseradish peroxidase, 10 mM sodium phosphate buffer and a 4:1 ratio of hydrogen peroxide to chlorogenic acid with the chlorogenic acid at: **gel 1**, no chlorogenic acid (CHLGA) no peroxide; **2**, 0.075 mM CHLGA; **3**, 0.15 mM CHLGA; **4**, 0.3 mM CHLGA; **5**, 0.6 mM CHLGA; **6**, 1.2 mM CHLGA; **7**, 2.4 mM CHLGA; **8**, 4.8 mM CHLGA; **9**, 9.6 mM CHLGA; **10**, 19.2 mM CHLGA; **11**, 19.2 mM CHLGA minus ribonuclease; **12**, 19.2 mM CHLGA minus horseradish peroxidase. **C,** The effect of the concentration and type of protein on crosslinking. Reaction mixtures contained 20 μg/ml of horseradish peroxidase, 2.4 mM hydrogen peroxide, 0.6 mM chlorogenic acid, 10 mM sodium phosphate buffer, pH 6.8, and the protein to be crosslinked at: **gel 1**, 2 mg/ml ribonuclease (RNase) minus horseradish peroxidase; **2**, 0.5 mg/ml RNase; **3**, 1.0 mg/ml RNase; **4**, 2.0 mg/ml RNase; **5**, 4.0 mg/ml RNase; **6**, 8.0 mg/ml RNase; **7**, 2 mg/ml lysozyme minus horseradish peroxidase; **8**, 2 mg/ml lysozyme; **9**, 2 mg/ml cytochrome c and buffer *only*; **10**, 2 mg/ml cytochrome c; **11**, 2 mg/ml bovine serum albumin (BSA) minus horseradish peroxidase; **12**, 2 mg/ml BSA; **13**, 1 mg/ml each BSA and RNase; **14**, 1 mg/ml each BSA and cytochrome c. **D,** Comparison of various phenolic substrates on crosslinking. The reaction mixtures contained 2 mg/ml ribonuclease, 20 μg/ml horseradish peroxidase, 2.4 mM hydrogen peroxide, 10 mM sodium phosphate buffer, pH 6.8, and 0.6 mM of the following: **gel 1**, *p*-hydroquinone; **2**, resorcinol (*m*-hydroquinone); **3**, catechol (*o*-hydroquinone); **4**, protocatechuic acid; **5**, *p*-hydroxycinnamic acid; **6**, caffeic acid; **7**, chlorogenic acid; **8**, L-dopa; **9**, epinephrine; **10**, 5-HO-tryptophan; **11**, tannin; **12**, esculin; **13**, naringen; **14**, rutin; and **15**, benzidine. Controls: **16**, ribonuclease, buffer and 2.4 mM periodate only; **17**, ribonuclease and buffer only. **E,** The components and reaction conditions necessary for crosslinking. The reaction mixtures were allowed to react for 20 hr at room temperature and contained the following: Gels 1–5 with 2 mg/ml ribonuclease, 20 μg/ml horseradish peroxidase (HRP), 2.4 mM hydrogen peroxide, 0.6 mM chlorogenic acid, and 10 mM sodium phosphate buffer, pH 6.8. **gel 1**, minus HRP; **2**, minus chlorogenic acid plus 0.6 mM L-ascorbate; **3**, no changes; **4**, plus 2.4 mM L-ascorbate; **5**, plus 300 units/ml catalase. Gels 6 & 7: **6**, 2.4 mM sodium periodate, ribonuclease and buffer only; **7**, same as gel 6, plus 0.6 mM chlorogenic acid. Gels 8–11 same as gels 1–5 substituting 1 unit of glucose oxidase per milliliter and 2.4 mM glucose for the hydrogen peroxide: **8**, minus HRP; **9**, minus HRP, plus 300 units of catalase per milliliter; **10**, no changes; **11**, plus 300 units of catalase per milliliter. Gels 12–15 same as gels 1–5 except substituting 2 mg/ml chyochrome c for ribonuclease; **12**, cytochrome c and buffer only; **13**, minus HRP; **14**, minus chlorogenic acid; **15**, no changes. **F,** The time course for crosslinking. The reaction mixtures contained the following: Gels 1–12: 2 mg/ml ribonuclease, 20 μg/ml horseradish peroxidase (HRP), 1.2 mM hydrogen peroxide, 0.6 mM chlorogenic acid, and 10 mM sodium phosphate buffer, pH 6.8. Reaction mixtures were allowed to react for: **1**, 0.5 min; **2**, 1 min; **3**, 2 min; **4**, 5 min; **5**, 10 min; **6**, 20 min; **7**, 30 min; **8**, 1 hr; **9**, 2.5 hr; **10**, 5 hr; **11**, 20 hr; **12**, 20 hr minus HRP. Gels 13–15 were allowed to migrate 1 cm further during electrophoresis. They contained the same reaction mixtures as in gels 1–12 except 1.5 μg/ml polyphenol oxidase in the presence of air was used instead of HRP and peroxide. Reaction mixtures were allowed to react: **13**, 5 min; **14**, 1 hr; **15**, 15 hr. Gels 16–18: reaction mixtures the same as in gels 1–12 with 2.4 mM sodium periodate instead of HRP and peroxide. Reaction mixtures were allowed to react: **16**, 5 min; **17**, 1 hr; **18**, 15 hr. **G,** Stability of the polymeric proteins and extended time course for crosslinking. Reaction mixtures contained 2 mg/ml ribonuclease, 20 μg/ml horseradish peroxidase (HRP), 1.2 mM hydrogen peroxide, 0.6 mM chlorogenic acid, and 10 mM sodium phosphate buffer, pH 6.8. Reaction mixtures were allowed to react 20 hr at room temperature and then 1 mM (final concentration) of sodium azide was added as a preservative. Reaction mixtures were then allowed to react further at room temperature a total of: **gel 1**, 1 day; **2**, 2 days; **3**, 4 days; **4**, 8 days; **5**, 16 days; **6**, 32 days; **7**, 1 day, ribonuclease and buffer only; **8**, 32 days, ribonuclease and buffer only. Gels 9–14 minus HRP and allowed to react: **9**, 1 day; **10**, 2 days; **11**, 4 days; **12**, 8 days; **13**, 16 days; **14**, 32 days.

Although catalase and HRP are both heme proteins and catalase under some conditions can also function as a peroxidase, no catalase preparation tested either oxidized the chlorogenic acid (no browning) or facilitated crosslinking in the absence of HRP.

Cytochrome c crosslinked well with: H_2O_2, chlorogenic acid, and HRP; H_2O_2 and chlorogenic acid without HRP; and H_2O_2 only (Fig. 3E, gels 13–15). Since cytochrome c is a heme protein, it is not unusual that it can act as a peroxidase, causing oxidation of the chlorogenic acid (browning). But since crosslinkage still occurred without addition of phenolic compounds, cytochrome c may be able to mediate radical reactions capable of crosslinking. One explanation could be the involvement of oxidized cytochrome c tyrosine residues (24); however, other radical reactions are possible. Of the proteins studied, cytochrome c was unique in its ability to be crosslinked in the absence of phenolic compound or peroxidase.

The time course for crosslinking. A study of the time necessary to optimally crosslink protein was carried out by the initiation of crosslinking by addition of 1.2 mM H_2O_2 under standard reaction mixture concentrations, and terminating the reaction by addition of the denaturing solution for SDS polyacrylamide gel electrophoresis. An increase in higher molecular weight oligomers was observed as early as 1 min after initiation (but not as early as 30 sec) and increased throughout the 24-hr incubation period (Fig. 3F, gels 1–11). Once initiated, the crosslinking reaction was slow with chlorogenic acid and, as will be seen in the next section, continued for weeks.

Polyphenol oxidase (1 unit per milliliter) in the presence of air as the oxidative system crosslinked well except that a longer period of time was necessary for equivalent crosslinkage to that obtained with peroxidase and H_2O_2 (Fig. 3F, gels 13–15). Sodium periodate (2.4 mM) also replaced the peroxidase and H_2O_2 as the oxidative system with a somewhat faster crosslinking reaction than with HRP and H_2O_2. However, by 15 hr a similar extent of crosslinkage had occurred with all three oxidative systems (Fig. 3F, gels 16–18). Thus, although the enzyme-mediated oxidation may be somewhat slower than direct chemical oxidation, the crosslinking reaction in itself was slow due to a rate-limiting chemical reaction rather than an enzymatic reaction. From earlier work in our laboratory, it was found that quinones generated by an oxidase can diffuse across a dialysis membrane and then oxidize and crosslink proteins on the other side of the membrane (26).

Stability of the polymeric protein and extended time course for crosslinking. The stability of the polymeric protein in the standard reaction mixture was studied by first letting it react for 20 hr and then by addition of 1 mM final concentration of either sodium azide or sodium benzoate as bacteriostatic agents for extended incubation at room temperature. Both preservatives are also reported to be peroxidase inhibitors (23). Crosslinking proceeded after day one and continued until at least day 32 (Fig. 3G) in the presence of either preservative. Marked increases in the number of bands and band density occurred throughout this period. Controls containing protein and buffer showed no change in bands over the entire period. In the standard reaction mixture minus the HRP, formation of a dimer slowly increased throughout the 32-day period. Since the H_2O_2 is unstable and should disproportionate in the first few days, air oxidation of chlorogenic acid probably contributed to crosslinkage. Since azide is a strong peroxidase inhibitor, it was clear that the oxidative system was only necessary to initiate the crosslinking reaction. Oxidized phenolics, once generated, were effective in crosslinking for a long period of time. Once crosslinked, the polymeric protein was stable in storage.

DISCUSSION

Protein polymers are probably formed by four different mechanisms involving the reactions of quinones or free radicals formed by the oxidative systems: (i) crosslinking by formation of a dehydrolysyl-nor-leucine link. This requires oxidation of an epsilon-amino group of a lysine residue to form lysylaldehyde that then condenses with a lysine residue in another protein to form a Schiff's base. Lysylaldehyde was reported to be formed when proteins are being crosslinked with a catechol and peroxidase-H_2O_2 oxidative system (26); (ii) a crosslink may be created when a quinone undergoes nucleophilic substitution by an amino or free sulfhydryl group of a protein, and the monosubstituted phenolic is again oxidized and undergoes a second nucleophilic substitution or

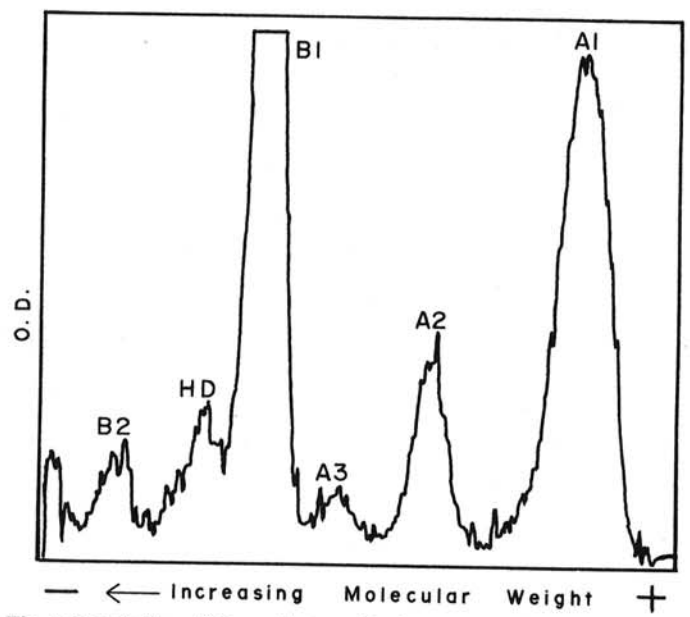


Fig. 4. Crosslinking of ribonuclease and bovine serum albumin (BSA). The densiometric trace of SDS gel number thirteen from Fig. 3C showed that ribonuclease (A1) and bovine serum albumin (B1) formed new polymeric bands consisting of the ribonuclease dimer (A2) and trimer (A3), and the bovine serum albumin-ribonuclease heterodimer (HD) when in the presence of oxidized chlorogenic acid. A BSA dimer (B2) was already present in the bovine serum albumin preparation.

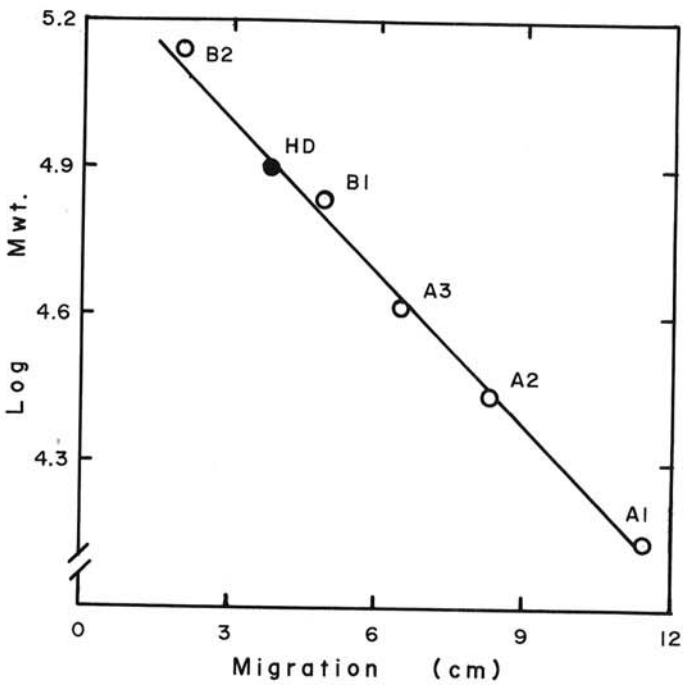


Fig. 5. Molecular weight of the bovine serum albumin-ribonuclease heterodimer. A plot of the logarithm of the theoretical molecular weight of each oligomer of ribonuclease (A1–A3) and bovine serum albumin (B1, B2) versus its observed migration on the densiometric trace in Fig. 4, gave a straight line. The molecular weights of ribonuclease and bovine serum albumin were assumed to be 13,700 and 69,000, respectively. When the logarithm of the heterodimer (HD, molecular weight 82,700) was plotted versus its observed migration, it fell on the line, consistent with the band being the heterodimer.

formation of a Schiff's base with another protein (18,21); (iii) a crosslink may be created through formation of a phenolic radical and its reaction with a side chain of an amino acid in a protein which then reacts with another phenolic-protein or protein to complete the covalent bond; and (iv) other radical attack creating a free radical on the protein which then reacts with a second protein radical to form a direct covalent crosslinkage (which may involve tyrosyl groups). It is expected that any or all of these mechanisms may contribute to the observed crosslinkage.

Since the quinone or semi-quinone probably directly participates in the crosslinking reaction, it was expected that different phenolics would crosslink to different extents under similar experimental conditions. The degree of crosslinkage also varied with the concentration of reactants. The observed optima for reaction components may be due to there being at least two competing reactions that are dependent on the concentration of quinone. The first that could lead to crosslinkage would be the reaction of a protein with the quinone; this has a rate equation dependent on the concentration of each. The second leads to polyphenol formation by the condensation of two quinones which has a rate equation dependent on the square of the quinone concentration. Since polyphenols, even at relatively low concentrations, can precipitate the protein, the situation is further complicated at high quinone concentrations. At low quinone concentrations the quinone becomes limiting in that there would not be enough to react with all of the protein.

Quinones, once formed, have interesting properties that may enable them to participate in disease resistance and in the formation of tissues that give structural support to plants and fungi. Depending on its chemical structure, a quinone may be rather stable and less reactive, or unstable and more reactive. A stable quinone might be advantageous in disease resistance because of its ability to diffuse through injured tissue and then react at a site distant from where it was generated. It could polymerize and/or inactivate hydrolytic enzymes (15,25) released by pathogens or even agglutinate pathogen cells. The quinones also may kill the pathogen and host cells in the region of the infection and polymerize with proteins or other substances to create an infection barrier around infection sites.

A very reactive quinone would be advantageous for strengthening structural components by reacting close to the site where generated, thus minimizing host cell damage. Quinones could crosslink proteins or phenolic esters linked to carbohydrates in plants (19) or chitosans in fungi (1). Quinones also can increase structural strength by forming polyphenols which are hydrophobic and can drive water out of the cell wall structure allowing crystallization of structural components such as may be the case with cellulose in secondary plant cell wall structure (20).

The concentrations of phenolics in plant tissue often greatly exceed the concentration of phenolics used in these model systems. Up to 3% dry weight rutin in buckwheat, 10% naringen in citrus peel rags, and 70% tannins in Chinese oak nut gall have been reported. However, the crosslinking reactions cannot occur until compartmentalized phenolic substrates and oxidative enzymes are brought into contact in the presence of an oxidant (O_2 or H_2O_2). In the case of plant disease resistance (infection barrier formation) this occurs when the plant tissue becomes structurally damaged. Since a great many phenolics are capable of participating in such crosslinking reactions, it is not surprising that there is so much diversity in the types of phenolics in plant and fungal tissue. However, there may be an advantage in using a certain type of phenolic compound in a given crosslinking reaction.

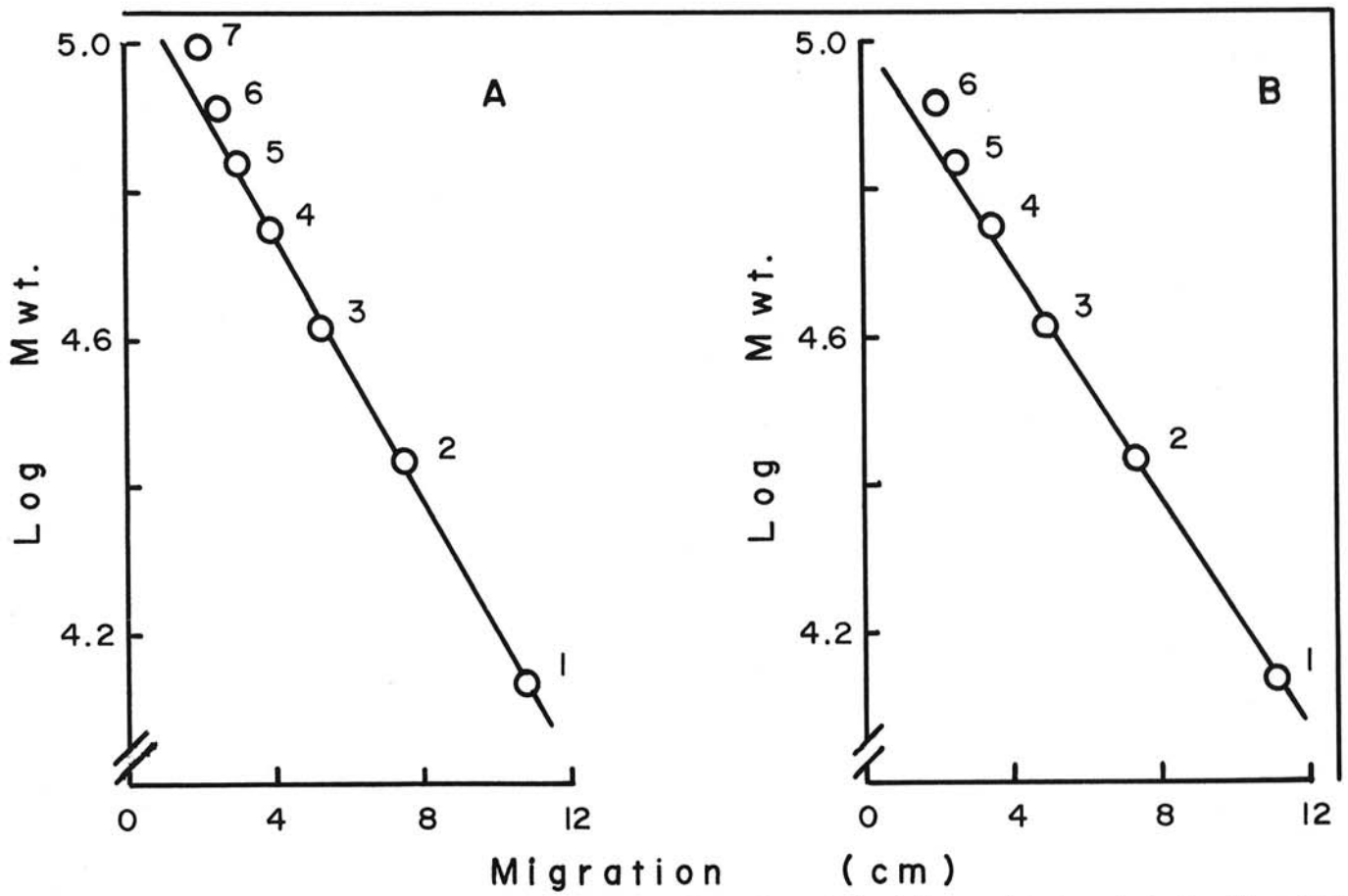


Fig. 6. Plots of the logarithm of the molecular weight of the expected ribonuclease polymers (points 1–7) versus their observed migration. The molecular weight of each oligomeric band was calculated from multiples of 13,700, the monomeric molecular weight of ribonuclease. The data were taken from densitometric tracings of gel #4, Fig. 3D, for protocatechuic acid (A); and gel #13, Fig. 3D, for naringen (B) as the crosslinking agents. The straight-line plots obtained again suggest that the bands were indeed oligomers of ribonuclease.

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