THE INTERACTION OF OXIDATION PRODUCTS OF PYROGALLOL WITH POLY-L-LYSINE. A MODEL OXIDATIVE BEER HAZE

by

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Iodate oxidation of pyrogallol at pH 4 produces an agent capable of precipitating poly-L-lysine and lysozyme. This substance is neither purpurogallin nor one of its oxidation products. The solubility of the poly-L-lysine precipitate in 6M-guanidine hydrochloride and in 1M-NaOH indicates that hydrogen bonding is responsible for precipitation. While the oxidized pyrogallol solutions do not contain tannins detectable by the method of CHAPON, polyvinylpyrrolidone (PVP) does prevent precipitation. Once formed, the precipitate does not redissolve in PVP. The pyrogallol oxidation products have no effect on the lysine-free polypeptide poly-N(5) hydroxyethyl-L-glutamine.

1. INTRODUCTION

Model systems for the formation of beer haze by oxidation have been extensively studied by CHAPON and coworkers who demonstrated that peroxidase catalyzed oxidation of beer rapidly produces haze (2) and that similar hazes can be formed by peroxidase catalyzed oxidation of polyphenols such as quercetin or catechin in the presence of acid-stable proteins such as ovalbumin or β -lactoglobulin (4). Changes in chromatographic behavior and haze activity due to peroxidase catalyzed oxidation of beer and of solutions of catechin or a synthetic procyanidin (1) indicate that oxidation leads to polymerization of the polyphenols, a moderate degree of polymerization producing tannins

capable of forming precipitates with proteins in beer while further polymerization decreases haze activity. Studies with PVP¹ (3) suggest that the tannin-protein reaction is reversible and that precipitation occurs when the protein forms a complex with a sufficient number of tannin molecules. The oxidative polymerization of monomeric polyphenols such as catechin to form tannins appears to involve condensations of *o*-quinones derived from the polyphenol (7).

Another possibility is that the quinones formed by polyphenol oxidation might condense with protein side chains such as the ϵ -amino groups of lysine. Such reactions occur rapidly in neutral solutions when catechol is oxidized by *o*-diphenol oxidase in the presence

of amino acids (8, 9, 11) and proteins (8, 12). The rates of irreversible condensation between *o*-quinones and ϵ -amino groups at the acid pH values (about 4) characteristic of beer should be considerably slower than the reaction at neutrality because of protonation of the amino groups in acidic solutions. To test whether such reactions might still be fast enough to compete with polyphenol polymerization and to assess the possible significance of *o*-quinone-amine condensations in beer haze formation, we have investigated the behavior of poly-L-lysine in a system in which pyrogallol is oxidized. While probably not an important agent in the formation of natural beer hazes, pyrogallol offers a much simpler model system for quinone reactions than such natural polyphenolic haze precursors as catechin. Preliminary studies were also performed with lysozyme – a protein rich in basic amino acids – and the lysine-free polypeptide PHEG.

2. MATERIALS AND METHODS

2.1. Materials

Analytic grade pyrogallol (E. Merck, Darmstadt, Germany) was sublimed in vacuo and stored in the dark in a tightly closed container. The absence of color in freshly prepared solutions showed that there was no oxidation of the crystals during storage or manipulation. Purpurogallin, prepared according to the methods of EVANS and DENN (5) and sublimed in vacuo, was bright orange and melted at 184–185°C (10°C/min heating rate). Aqueous solutions of purpurogallin were prepared by diluting 0.300 mg/ml methanolic stock solutions. Poly-L-lysine (mol.wt.41,000) and lysozyme (3x recrystallized) were from Sigma (St. Louis, Mo.). Concentrations of stock solutions of these two reagents were determined by amino acid analysis (section 2.3). The Luviskol K90 preparation of PVP (mol.wt. 700,000) was a gift from J. A. SØRENSEN. Poly-N(5)hydroxyethyl-L-glutamine (PHEG-4) was a gift from Dr. NERI, Sclavo Co., Siena, Italy. Guanidine hydrochloride (puriss.) was from Fluka AB, Switzerland, and potassium iodate was from

Struers, Copenhagen. All other reagents were analytic grade from E. Merck, Darmstadt.

2.2. Spectra

Ultraviolet-visible spectra of oxidizing systems were recorded from 225–700 nm using a Pye-Unicam, SP 800 ultraviolet spectrophotometer (Cambridge, England). The reference cell contained the same acetate buffer used in the oxidation reaction.

2.3. Amino acid analysis

Amino acid analyses of haze precipitates, supernatant solutions and stock solutions of poly-L-lysine and lysozyme were performed after 24 hours hydrolysis with 6M-HCl at 110°C in sealed ampoules. Hydrolysates were analyzed either with a Beckman Spinco amino acid analyzer (Palo Alto, Calif.) with a 12 cm Aminex column using pH 5.28 citrate buffer or with a Durrum Model D 500 amino acid analyzer (Palo Alto, Calif.) using the standard procedure in the Durrum Manual. The same ninhydrin development method was used in both procedures. The mass of poly-L-lysine was estimated by multiplying the moles of lysine found by the mass of a lysine residue (128).

2.4. Thin layer chromatography

Thin layer chromatography of oxidation was attempted using silica gel 60F₂₅₄ on 0.25 mm plastic sheets (E. Merck, Darmstadt) or polyamide on plastic (BDH Chemicals, Ltd., Poole, England) as the stationary phase. The polyamide sheets were soaked in a 250 mg/l solution of 2-(*tert*-butylphenyl)-5-(4-biphenyl)1,3,4-oxadiazol (Fluka, Switzerland) and dried before use, and the chromatograms were analyzed by means of a Mineralight UVS-11 lamp (Ultraviolet Products, San Gabriel, Calif.). Moving phases were: 1) water-ethanol-ethyl acetate (4:2:1 v/v); 2) water-methanol (1:1); 3) 10% acetic acid in chloroform (v/v).

2.5. The model haze system

To 3.0 ml of an acetate buffer (*I* = 0.10, pH 3.98 to 4.09 depending on the experiment) maintained at 25°C in a 1.0 cm silica cuvette,

1) Abbreviations: PVP, polyvinylpyrrolidone, PHEG, Poly-N(5)hydroxyethyl-L-glutamine

were added 50 μ l of the appropriate freshly prepared pyrogallol stock solution and 250 μ l of poly-L-lysine stock solution. In some experiments, the poly-L-lysine was dissolved directly in the buffer. Addition of potassium iodate solution, varying in concentration from 0.047 to 0.062 M and in volume from 10 to 130 μ l, initiated the oxidation. Spectra were recorded from 450 to 225 nm, initially at two minute intervals, then less frequently as the reaction rate decreased. After 30 minutes, the spectrum was periodically scanned from 700 to 315 nm. In several experiments, the haze was centrifuged after two or three hours for twenty minutes at 2,000 rev./min, the precipitate washed once with one ml water and re-centrifuged. Amino acid analyses were performed on a suspension of the precipitate in one ml water, on the poly-L-lysine stock solution and, in some cases, the untreated supernate.

The lysozyme haze was formed by a reaction between 3.0 ml of 1 mg/ml lysozyme in pH 4.09 acetate buffer, 50 μ l 0.090 M-pyrogallol and 130 μ l 0.055 M-pyrogallol and 130 μ l 0.055 M-KIO₃. The intermediate spectra were not recorded, but the precipitate was recovered and analyzed in the same way as the poly-L-lysine precipitate.

Variations in the experimental procedure involving control experiments, the investigation of the influence of time of addition of poly-L-lysine, the effects of PVP, PHEG and purpurogallin, and variations in the KIO₃-pyrogallol ratio are described in section 3.

2.6. Stoichiometry of oxidation

A 0.0100 M-solution of sodium thiosulfate pentahydrate was standardized by mixing 1.0 ml of 0.00188 M-potassium iodate in pH 4.09 acetate buffer, 0.10 ml 1 M-HCl and 1.0 ml 0.033 M-sodium iodide and titrating with the thiosulfate to a starch end point. To determine the stoichiometry of the iodate-pyrogallol reaction, 1.0 ml 0.0353 M-pyrogallol and 1.0 ml 0.0470 M-KIO₃ were added to 23.0 ml 0.10 M-acetate buffer, pH 4.09. Aliquots (1.0 ml) were removed at various times, mixed with 0.10 ml 1 M-HCl and 20 μ l 1% starch and titrated with thiosulfate. Iodate consumption ceased after one hour.

MOLECULAR STRUCTURES

3. RESULTS

3.1. Spectroscopic changes during oxidation

During the first ten minutes of oxidation of 0.21 mM-pyrogallol (I) by 0.74 mM-potassium iodate at pH 3.98, weak extinction bands appear at 275, 305 and 400 nm while strong extinction develops below 235 nm (Fig. 1a). The 275 and 305 nm maxima, which are due to purpurogallin (II), disappear during the next hour, and there is also a decrease in extinction at 400 nm and a further increase in extinction at low wavelengths and in the 325-370 nm range. There are no spectroscopic changes after 90 minutes.

When the pyrogallol-iodate ratio is approximately 2:1 (0.32 mM-pyrogallol, 0.15 mM-KIO₃), the stoichiometric ratio for the oxidation of pyrogallol to purpurogallin, the 275 and 305 nm peaks do not decay, and a third weak purpurogallin shoulder at 375 nm is apparent. Although the extinction at 400 nm is stronger than for pure purpurogallin (Fig. 1b), the maximum at 400 nm is not present. The presence of strong extinction at low wavelength shows that purpurogallin is not the sole oxidation product. Based on an estimated extinction coefficient for purpurogallin at 305 nm in pH 4.09 acetate buffer of 2.1×10^4 liter-mole⁻¹ cm⁻¹, the estimated yield of purpurogallin is at most 20%.

An experiment in which 0.060 mM-purpurogallin was oxidized in 0.14 mM KIO₃ (Fig. 1b) shows that the decay of the 275 and 305 nm extinction maxima in the presence of excess KIO₃ is due to further oxidation of purpurogallin. There is a decrease in extinction between 265 and 335 nm and slight increases below 240 nm and above 335 nm. These increases are not sufficient to explain the strong extinction below 235 nm or the transient extinc-

tion at 400 nm when pyrogallol is oxidized in excess KIO_3 .

3.2. Stoichiometry of oxidation

When 1.41 mM-pyrogallol is oxidized in the presence of 1.88 mM- KIO_3 in pH 4.09 acetate buffer, 0.76 mole iodate is consumed for every mole pyrogallol. This is considerably larger than the 0.50 ratio required for purpurogallin formation.

3.3. Thin layer chromatography

None of the combinations of stationary and moving phases used adequately resolves the oxidation product mixture. In all cases, solutions that had been oxidized for at least one hour contained only material with low R_F values. The greatest movement was observed with 10% acetic acid in chloroform on silica gel which gave a continuous streak from R_F 0 to 0.32. All the material detectable by use of the fluorescent indicator was also yellow-brown.

3.4. Haze information

Poly-L-lysine concentrations ranging from 6 to 600 $\mu\text{g/ml}$ have no influence on the spectra of solutions in which iodate oxidizes pyrogallol at pH 4. However, turbidity is apparent, both visually and by increased apparent extinction above 500 nm due to light scattering, after about one hour. The dark red-brown precipitate responsible for the turbidity can be centrifuged easily and coagulates over 24 hours. This precipitate is insoluble in 1 M-HCl, methanol and carbon tetrachloride but dissolves readily in either 1 M-NaOH or in 6 M-guanidine hydrochloride. The NaOH solution of the precipitate has strong extinction below 235 nm but no maxima at higher wavelengths. No turbidity or precipitate results from the action of either pyrogallol or KIO_3 alone on poly-L-lysine.

Poly-L-lysine need not be present during the initial stages of oxidation to produce a precipitate. Precipitates were formed when 250 μl of 600 $\mu\text{g/ml}$ poly-L-lysine was added to 3.0 ml of solutions in which 0.26 mM-pyrogallol had been oxidized by 1.10 mM- KIO_3 for as long as 21 hours. The relative amounts of polyphenolic material precipitated at the various times were

Spectroscopic Changes

Figure 1. Spectroscopic changes during pyrogallol oxidation.

1a. — 0.21 mM pyrogallol oxidized by 0.74 mM potassium iodate at pH 3.98 for 12 minutes; ---- oxidized for 160 minutes; - - - - 0.32 mM-pyrogallol oxidized by 0.15 mM-potassium iodate presence of 33 $\mu\text{g/ml}$ poly-L-lysine at pH 4.09 for 120 minutes.

1b. — Spectrum of 0.060 mM-purpurogallin in the presence of 33 $\mu\text{g/ml}$ poly-L-lysine at pH 4.09; ---- after oxidation with 0.14 mM-potassium iodate for 200 minutes.

1c. — Spectrum of 0.20 mM-pyrogallol oxidized by 0.68 mM-potassium iodate in the presence of 75 $\mu\text{g/ml}$ PVP and 0.39 mg/ml poly-L-lysine at pH 4.01 for 10 minutes; ---- oxidized for 165 minutes.

Table I

Yields of Haze Precipitate

pyrogallol concentration (mM)	poly-L-lysine concentration ($\mu\text{g/ml}$)	μg poly-L-lysine precipitated per ml solution	poly-L-lysine concentration in supernate ($\mu\text{g/ml}$)
0.20	600	2.0	----
0.21	60	1.1	----
0.18	6	0.9	----
1.35	37	approx. 10	10
1.20	410	19	357

estimated by redissolving the precipitates in 1 M-NaOH and measuring the extinction at 325 nm. This experiment indicates that the amount of polyphenol precipitated is independent of the time of addition of the poly-L-lysine. Late addition of poly-L-lysine does produce a more flocculent precipitate and a less stable haze.

The data in Table I show that the amount of poly-L-lysine precipitated is limited by the amount of pyrogallol present. At a given pyrogallol concentration, the concentration of poly-L-lysine has very little influence on the amount of polypeptide precipitated. On the other hand, the amount of poly-L-lysine precipitated is roughly proportional to the pyrogallol concentration. Attempts to study the reaction with 9.7 mM-pyrogallol were foiled by the precipitation of a polyphenolic oxidation product, most probably purpurogallin.

The haze is not due to purpurogallin or its oxidation products. There was no apparent turbidity after an hour's contact between 32 $\mu\text{g/ml}$ poly-L-lysine and 0.060 mM-purpurogallin at pH 4.09 nor did oxidation of this solution with 0.14 mM-KIO₃ produce a precipitate.

3.5 The effect of PVP

The presence of PVP during the early stages of pyrogallol oxidation alters the spectrum of the solution (Fig. 1c) by producing persistent extinction in the 325-375 nm range. The effect is not due to the interaction of PVP with either pyrogallol or KIO₃ alone. PVP has no effect on the spectrum if it is added after oxidation is complete. No turbidity or precipitate forms during the oxidation of pyrogallol in the presence of either 0.078 or 1.0 mg/ml PVP, nor

could any tannins be detected in an oxidized solution of 1.15 mM-pyrogallol by the PVP method of CHAPON (3). In the tannin analysis, the PVP concentration was slowly increased to 0.068 mg/ml without producing detectable turbidity.

The presence of PVP in concentrations as low as 0.078 mg/ml prevents precipitation when 0.20 mM-pyrogallol is oxidized in the presence of 390 $\mu\text{g/ml}$ poly-L-lysine, nor does precipitation occur when 1.20 mM-pyrogallol is oxidized in the presence of 29 $\mu\text{g/ml}$ poly-L-lysine and 0.071 mg/ml PVP. Addition of PVP after completion of oxidation but before addition of poly-L-lysine also prevents precipitation. However, 0.078 mg/ml PVP does not redissolve the precipitate once it forms.

3.6. Lysozyme haze

A thick haze forms after eight minutes oxidation of 1.4 mM-pyrogallol by 2.2 mM-KIO₃ in the presence of 1 mg/ml lysozyme at pH 4.09. This red-brown precipitate coagulates more readily than the poly-L-lysine haze. Like the poly-L-lysine haze, it dissolves readily in 6M-guanidine hydrochloride.

3.7 The effect of PHEG

There are no spectroscopic effects due to the presence of approx. 0.1 mg/ml PHEG when 0.21 mM-pyrogallol is oxidized at pH 4.09, nor does PHEG prevent precipitation in the presence of 37 $\mu\text{g/ml}$ poly-L-lysine. PHEG (0.1 mg/ml) does not form a precipitate or turbidity with the oxidation products of pyrogallol, even at pyrogallol concentrations as high as 1.24 mM.

4. DISCUSSION

Oxidation of pyrogallol at pH 4 produces, among other products, one or more substances capable of precipitating poly-L-lysine and lysozyme. This precipitating agent is not purpurogallin, which is formed in the oxidation reaction, or further oxidation products of purpurogallin. The lack of influence of poly-L-lysine on the rate of oxidation or the spectroscopic changes due to oxidation and the fact that the precipitating agent persists long after oxidation has ceased indicates that oxidation intermediates such as monomeric quinones or semiquinones are not involved in the precipitation. Based on the spectra of fully oxidized solutions and the precipitate redissolved in NaOH, the precipitating agent seems to have strong extinction below 235 nm and a featureless spectrum at higher wave lengths. Polymeric oxidation products such as biphenyls (6) or triphenoquinones (10) might be the precipitating agents.

The formation of the precipitate seems to involve hydrogen bonding since the precipitate dissolves both in guanidine hydrochloride and in 1M-NaOH. The first reagent is effective in disrupting all types of hydrogen bonds while the second prevents hydrogen bond donor activity by phenols by deprotonating them. There was no indication in the amino acid analyses of formation of non-hydrolyzable covalent condensation products between the ϵ -amino groups of poly-L-lysine and the polyphenol oxidation product.

The oxidation product that precipitates poly-L-lysine and lysozyme is selective in its interactions since it does not cause turbidity or precipitation with PVP or PHEG, two polymers capable of extensive hydrogen bonding. As shown by its effect on the oxidation spectrum (Fig. 1c), PVP does interact with oxidation intermediate, and its ability to prevent poly-L-lysine precipitation when added to a fully oxidized solution shows that it complexes with the precipitating agent. These interactions do not, however, precipitate PVP.

According to the criterion of CHAPON (3) the oxidized solution contains no detectable amount of tannins. One possible explanation of the selectivity of the precipitating agent and its

apparent inability to act as a tannin is that it might contain carboxyl groups due to ring cleavage reactions (13). Such molecules could complex with PVP, but their negative charges would prevent precipitation of the complex. On the other hand, a negatively charged precipitating agent would neutralize the positive charges of poly-L-lysine and lysozyme and thus aid precipitation.

The precipitation of poly-L-lysine is not readily reversible since PVP, which is effective in preventing precipitation, does not redissolve the precipitate. This may be due to a relatively slow conformational change of an initially formed polyphenol-poly-L-lysine complex to produce an insoluble, tightly bound complex.

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REFERENCES

1. CHAPON, L., S. CHAPON, R. SEYER & C. LOUIS: Experiences modeles illustrant quelques aspects du mecanisme de formation du trouble au froid. Proc. Eur. Brewery Conv., 15th Congress, Nice 1975, Elsevier, Amsterdam pp. 423-438 (1975)
2. CHAPON, L., B. CHOLLOT & E. URION: Oxydation peroxydasique des bières. Phénomènes fondamentaux. Proc. Eur. Brewery Conv., 8th Congress, Vienna 1961, Elsevier, Amsterdam pp. 319-333 (1961)
3. CHAPON, L., B. CHOLLOT & E. URION: Étude physico-chimique des associations entre protéines vegetales et substances polyphenoliques. Bull.Soc.Chim. Biol. 43, 429-442 (1961)
4. CHOLLOT, B., L. CHAPON & E. URION: Précurseurs polyphénoliques et protéiques du trouble d'oxydation peroxydasique. Proc. Eur. Brewery Conv., 8th Congress, Vienna 1961, Elsevier, Amsterdam pp. 334-350 (1961)
5. EVANS, T. W. & W. M. DENN: Organic oxidations by iodic acid. J. Am. Chem. Soc. 52, 3647-3649 (1930)
6. HARRIES, C.: Über die Autoxydation des Pyrogallols. Chem.Ber. 35, 2954-2959 (1902)
7. HATHWAY, D. E. & J. W. T. SEAKINS: Autoxidation of catechin. Nature 176, 218 (1955)

8. MASON, H. S.: Reactions between quinones and proteins. *Nature* 175, 771-772 (1955)
9. MASON, H. S. & E. W. PETERSON: Melanoproteins I. Reactions between enzyme-generated quinones and amino acids. *Biochim. Biophys. Acta* 111, 134-146 (1965)
10. NIERENSTEIN, M.: An oxidation product of pyrogallol. *J. Chem. Soc.* 107, 1217-1220 (1915)
11. PIERPOINT, W. S.: *o*-Quinones formed in plant extracts. Their reactions with amino acids and peptides. *Biochem. J.* 112, 609-617 (1969)
12. PIERPOINT, W. S.: *o*-Quinones formed in plant extracts. Their reactions with bovine serum albumin. *Biochem. J.* 112, 619-629 (1969)
13. SCHULZE, H. & W. FLAIG: Zur Kenntnis der Huminsäuren, IV. Mitteilung. Über die Ringsprengung mehrwertiger Phenole mit Sauerstoff in alkalischem Medium. *Ann.* 575, 231-241 (1952)