STUDIES ON THE ENZYMIC BROWNING OF APPLES

II. PROPERTIES OF APPLE POLYPHENOLOXIDASE

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Summary

The properties of a particulate polyphenoloxidase preparation from young apple fruit have been investigated. Chlorogenic acid, D-catechin, and hydrocaffeic acid were found to be the substrates most rapidly oxidized by this preparation, but its action on a number of other phenolic compounds was also investigated. The inhibition of the oxidation of phenolic compounds by cysteine and other thiol compounds was studied in detail.

I. INTRODUCTION

Evidence that the enzyme polyphenoloxidase (PPO) is of major importance in catalysing the browning of apples and other fruit has been reviewed by Hulme (1958). Weurman and Swain (1953) and also Hulme (1958) have suggested that chlorogenic acid (3,4-dihydroxyphenyl-3-quinic acid) is a major substrate for this enzyme in apple and pear fruit. Phenolic compounds such as p-coumarylquinic acid (Williams 1958), catechin, and l-epicatechin (Nakabayashi 1953; Williams 1953) have also been detected, but their role in the biochemistry of the fruit is not yet clearly established although Seigelman (1955) considers l-epicatechin to be the principal substrate for PPO in apple and pear skins.

The browning of apple tissue resulting from the action of PPO is not only of fundamental interest. It may also be involved in the development of physiological disorders of apples, such as “bitter pit”, and affects the quality of processed apple products.

II. EXPERIMENTAL

Earlier studies at this Institute on the browning of local apple varieties (Walker 1962) indicated that the cultivar Sturmer Pippin showed a high level of enzymic browning, and since this apple stores well and is an important export variety it was chosen for study. Young apple fruitlets were used as a source of enzyme since they exhibit a high degree of browning and have a low starch content. Fruitlets were gathered 6 weeks after petal drop, stored in sealed plastic bags at 2°C, and used as required. Later confirmatory experiments with mature fruit gave virtually identical results but involved a tedious separation of the enzyme particles from starch grains.

(a) Preparation of Enzymically Active Material

Apple fruitlets (100 g) were peeled, quartered, and dropped into 200 ml of ice-cold buffer (0.2M Na₂HPO₄ plus 0.005M cysteine hydrochloride) contained in a

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pre-chilled blender jar. After disintegration the resulting homogenate was filtered through a double layer of fine cloth, then centrifuged at 7000 g at 4°C in an International HR-1 refrigerated centrifuge for 15 min. The resultant green precipitate was washed three times with 1% KCl solution and finally taken up in 30 ml 1% KCl solution. Typical preparations contained 400–500 µg nitrogen/ml and 1·5–2·0 µg chlorophyll/ml (estimated by the method of Arnon 1949). Since Mayer and Friend (1960a) found PPO activity in the chloroplast, mitochondrial, and soluble fractions of homogenates of sugar-beet leaves, the supernatant from the first centrifugation step was respun at 34,500 g for 30 min at 4°C, then treated with 3 volumes cold (−15°C) acetone, but no more enzymically active material was recovered.

Examination of the green precipitate with the optical microscope showed it to consist of small, more or less spherical, particles about 1 µ in diameter. From their centrifugal and microscopical behaviour it was deduced that they were chloroplast fragments or grana. This preparation could be stored for at least 2 weeks at 4°C with no significant loss of enzymic activity. Attempts were also made to isolate whole chloroplasts from young fruit by disintegration in 0·5M sucrose solution, but the harsh mechanical treatment needed to break up the hard fruitlet tissue inevitably caused the rupture of most of the chloroplasts.

(b) Preparation and Properties of Soluble Apple PPO

In view of reports by several workers (Clayton 1959; Björkman and Holmgren 1960) of the existence of separate chlorogenic acid and catechol oxidases in certain plants, attempts were made to solubilize and fractionate the apple PPO used in the present studies.

A suspension of chloroplast fragments, prepared as already described, was treated with 2 volumes of cold (−15°C) acetone and then washed with a further quantity of cold acetone to remove chlorophyll. The resultant buff-coloured precipitate was treated with 1% KCl, 0·1M phosphate buffer (pH 7·2), and detergents (Mayer and Friend 1960b), but no PPO activity could be extracted. However, PPO activity was extracted by Na2CO3 (0·1M)–cysteine (0·005M) solution (Ponting and Joslyn 1948). The extract, after clarification by centrifuging at 34,000 g for 20 min, was light orange-brown in colour and had a pH of 10·6. If the pH of this extract was lowered by addition of solid KH2PO4, or by treatment with a cation-exchange resin [IR-120 (H+ form)], or if cold acetone was added, a buff-coloured precipitate with PPO activity reappeared. This precipitate was soluble in solutions only of pH greater than 9·6 which severely limited the scope of subsequent fractionation procedures.

Chromatographic separation of the enzyme was attempted by applying bands of the alkaline extract to strips of DEAE-cellulose and Ecteola cellulose ion-exchange papers and, after development with Na2CO3–cysteine solution, the strips were sprayed with dilute (0·02M) solutions of chlorogenic acid, catechol, or dihydroxyphenylalanine (DOPA) to locate the enzyme. Only one very slow-moving band was found in each case. Similar results were obtained by paper electrophoresis (pH 8·6, borate buffer) using an EEL apparatus; only one slow-moving band of protein, coincident with PPO activity, was observed.
When a 5-ml portion of the soluble PPO preparation was applied to a column (20 x 2 cm) of Sephadex G-100 and the column eluted with \( \text{Na}_2\text{CO}_3 \)-cysteine solution, resolution into two orange-brown bands was observed. The faster-moving band was eluted in the void volume and contained all the original PPO activity. The second slower-moving band was inactive and darkened on standing. No such separation occurred on similar columns of Sephadex CM-50 (cation exchanger) whilst the enzyme remained firmly bound on Sephadex A-50 (anion exchanger).

The ratio of enzyme activity towards chlorogenic acid, catechol, and \( p \)-cresol remained the same before and after the above fractionation procedures, namely 100 : 40-45 : 7-9, respectively. Behaviour towards inhibitors also remained unaltered. It was therefore assumed that only one enzyme was present in the original particulate PPO preparation which was therefore used in all subsequent experiments.

![Graph](image)

**Fig. 1.**—Thermal inactivation of a polyphenoloxidase preparation from apples. Reaction mixtures (3·0 ml) contained 10 \( \mu \)moles chlorogenic acid, 0·5 ml enzyme, 0·5 ml 1% gelatine, and 0·1m phosphate-citrate buffer (pH 5·0). (a) Enzyme held at different temperatures for 5-min periods. (b) Enzyme held at 70°C for different times.

**Reagents**

Chlorogenic acid, isochlorogenic acid, and hydrocaffeic acid were obtained from Fluka AG, Switzerland. Other phenolic compounds were purchased from L. Light & Co Ltd., England, and purified by appropriate recrystallization procedures. DL-Homocysteine was prepared by treating DL-homocysteine thiolactone hydrochloride with 2N \( \text{NH}_4\text{OH} \) and then removing excess ammonia under vacuum. Aesculetin was made by hydrolysing aesculin with 2N HCl.

**Measurement of Oxygen Absorption**

This was done by conventional Warburg techniques at 30°C and in an atmosphere of air. Each flask contained 0·1m phosphate-citrate buffer, pH 5·0 (MacIlvaine 1921), 0·5 ml 1% gelatine solution, together with enzyme and inhibitors
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Substrates were added from the side-arm after equilibration. The total volume of fluid in each flask was adjusted to 3 ml in all experiments.

Check experiments using flasks with KOH in the centre well to absorb any CO₂ formed showed oxygen uptakes identical with those without KOH.

![Graph showing oxygen uptake over time for different substrates.](image)

Fig. 2.—Oxidation of phenolic compounds by an apple polyphenoloxidase preparation. The reaction mixture (total vol. 3 ml) contained enzyme, 0·5 ml 1% gelatine, 0·1 ml phosphate-citrate buffer (pH 5·0), and 10 μmoles substrate, as follows: A, chlorogenic acid (brown); B, hydrocaffeic acid (bright red); C, catechol (dark brown); D, isochlorogenic acid (light brown); E, d-catechin (orange); F, 3,4-dihydroxyphenylacetic acid (light brown); G, L-3-(3,4-dihydroxyphenyl)alanine (dark red); H, p-cresol (red-brown); I, caffeic acid (brown); J, 3,4-dihydroxybenzoic acid (light brown); K, no substrate (colourless). The colours refer to those of the final reaction mixtures after centrifuging.

III. RESULTS

(a) Determination of Optimum pH for Enzyme Activity

The initial rate of oxygen absorption when apple PPO was incubated with 10 μmoles chlorogenic acid in phosphate-citrate buffer at various pH values in the range 3·5–7·0 was measured. Maximum activity occurred at pH 5·0; therefore subsequent experiments were carried out at this pH.
(b) Thermal Inactivation of Apple PPO

Because of the local importance of processed apple products the thermal inactivation of apple PPO was investigated. The results of this experiment [Fig. 1(a)] show that inactivation first became marked at 70°C and that activity was destroyed at 80°C. The effect of time of heating at 70°C was also investigated [see Fig. 1(b)]; at this temperature the enzyme was found to have a half-life of approximately 12 min. Similar results were obtained with an enzyme preparation from mature fruit.

![Graph](image_url)

Fig. 3.—Effects of enzyme concentration on oxidation of phenolic compounds. Warburg flasks contained enzyme, 0.5 ml 1% gelatine, 0.1 M phosphate-citrate buffer (pH 5.0), and 10 μmoles substrate in a total volume of 3 ml: A, chlorogenic acid; B, hydrocaffeic acid; C, catechol; E, p-catechin; G, L-3(3,4-dihydroxyphenyl)alanine; I, caffeic acid. (a) Relative rates of oxidation; (b) amount of oxygen finally absorbed; (c) optical density (at 490 mμ) of final flask contents diluted to 10 ml.

(c) Substrate Specificity of Apple PPO

In order to try to find out which phenolic compounds were the most favourable substrates for apple PPO the enzyme was incubated with 10 μmoles of each of the phenolic substances listed in the legend to Figure 2, which also records the colours of the final reaction mixture. The results indicate that chlorogenic acid, p-catechin, catechol, and hydrocaffeic acid were all rapidly oxidized, although the latter two compounds do not normally occur in apples. The following compounds were also tested but were not oxidized, neither did they yield coloured products at the end of the reaction period (60 min): cinnamic acid, hydrocinnamic acid, o-hydroxyphenylacetic acid, ferulic acid, sinapic acid, p-hydroxybenzoic acid, gentisic acid (2,5-dihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid, syringic acid, phenylalanine, tyrosine, quercetin, quercitrin, rutin, and aesculin.
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When preparations of apple PPO were incubated with \( p \)-coumaric acid for longer reaction times (4 hr) oxygen was absorbed and the contents of the reaction flask became light brown; subsequent examination by paper chromatography revealed the presence therein of caffeic acid.

(d) Effect of Enzyme Concentration

Figure 3 shows the results of different amounts of apple PPO with various substrates. Although the rate of oxidation was proportional to enzyme concentration this relationship was not always observed for final oxygen absorption or colour production. Caution must therefore be exercised if PPO is assayed by measurement of coloured oxidation products. In the case of catechol and DOPA total oxygen consumption was dependent on enzyme concentration.

![Figure 4](image-url)

Fig. 4.—Effect of reaction products on the oxidation of chlorogenic acid by a polyphenoloxidase preparation from apples. Double-side-arm Warburg flasks contained 0.5 ml 1% gelatine and 0.1M phosphate-citrate buffer (pH 5.0) in 3 ml solution. Flask A contained 10 \( \mu \)moles chlorogenic acid and 0.2 ml enzyme. Flask B contained 10 \( \mu \)moles chlorogenic acid plus 0.1 ml enzyme; a further 0.1 ml enzyme was added later from the second side-arm. Flask C contained 6 \( \mu \)moles chlorogenic acid and 0.2 ml enzyme; extra substrate (4 \( \mu \)moles) added from the second side-arm. The enzyme preparation used in this experiment was more dilute (1 : 5) than that used for other experiments.

(e) Effect of Substrate Concentration

Attempts to determine the Michaelis constant of apple PPO for the direct oxidation of chlorogenic acid were unsuccessful because of the rapid inactivation of the enzyme by its reaction products. Resort was therefore made to the coupled oxidation of ascorbic acid by phenolic compounds: a reaction used by several workers (e.g. Sreerangachar 1943; Arnon 1949; Sisler and Evans 1958) for assaying PPO preparations. Different amounts of the phenolic substrate were incubated...
TABLE 1

EFFECT OF INHIBITORS ON THE RATE OF OXIDATION OF CHLOROGENIC ACID BY A POLYPHENOLOXIDASE PREPARATION FROM APPLES

Warburg flasks contained 10 µmoles chlorogenic acid, inhibitor, 0·1 ml enzyme, 0·5 ml 1% gelatine, 0·1M phosphate–citrate buffer (pH 5·0) in 3 ml solution at 30°C

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (M)</th>
<th>Rate of Oxygen Absorption (µl/min)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>34·5</td>
<td>0</td>
</tr>
<tr>
<td>Sodium diethyldithiocarbamate</td>
<td>10⁻³</td>
<td>5·2</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁻⁴</td>
<td>6·0</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>8·1</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>28·6</td>
<td>17</td>
</tr>
<tr>
<td>2,3-Dimercaptopropan-1-ol (BAL)</td>
<td>10⁻³</td>
<td>4·0</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>29·0</td>
<td>16</td>
</tr>
</tbody>
</table>

TABLE 2

ACTION OF THIOL COMPOUNDS ON THE OXIDATION OF CHLOROGENIC ACID BY A POLYPHENOLOXIDASE PREPARATION FROM APPLES

Warburg flasks contained 8 µmoles chlorogenic acid, 0·1 ml enzyme, 0·5 ml 1% gelatine, 0·1M phosphate–citrate buffer (pH 5·0), added thiol compounds, in 3 ml solution at 30°C

<table>
<thead>
<tr>
<th>Thiol Compound</th>
<th>Amount (µmoles)</th>
<th>Final Oxygen Absorbed (µmoles)</th>
<th>Colour of Final Reaction Mixture</th>
<th>Optical Density at 490 mµ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>5·2</td>
<td>Brown</td>
<td>0·25</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>4</td>
<td>4·1</td>
<td>Brown</td>
<td>0·27</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3·5</td>
<td>Red-brown</td>
<td>0·11</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1·7</td>
<td>Colourless</td>
<td>0·0</td>
</tr>
<tr>
<td>Glutathione</td>
<td>4</td>
<td>5·1</td>
<td>Brown</td>
<td>0·26</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2·5</td>
<td>Colourless</td>
<td>0·0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1·9</td>
<td>Colourless</td>
<td>0·0</td>
</tr>
<tr>
<td>DL-Homocysteine</td>
<td>4</td>
<td>5·2</td>
<td>Brown</td>
<td>0·25</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4·0</td>
<td>Brown</td>
<td>0·20</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1·9</td>
<td>Colourless</td>
<td>0·0</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>4</td>
<td>5·1</td>
<td>Brown</td>
<td>0·26</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5·1</td>
<td>Brown</td>
<td>0·25</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2·1</td>
<td>Colourless</td>
<td>0·0</td>
</tr>
<tr>
<td>DL-Penicillamine</td>
<td>4</td>
<td>4·9</td>
<td>Brown</td>
<td>0·27</td>
</tr>
<tr>
<td>(β,β’-dimethylcysteine)</td>
<td>8</td>
<td>4·7</td>
<td>Brown</td>
<td>0·27</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2·2</td>
<td>Colourless</td>
<td>0·0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>4</td>
<td>5·2</td>
<td>Brown</td>
<td>0·26</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5·2</td>
<td>Brown</td>
<td>0·25</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>4</td>
<td>5·1</td>
<td>Brown</td>
<td>0·25</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5·0</td>
<td>Brown</td>
<td>0·25</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>4</td>
<td>3·6</td>
<td>Brown</td>
<td>0·26</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>3·7</td>
<td>Brown</td>
<td>0·26</td>
</tr>
<tr>
<td>Thiourea</td>
<td>4</td>
<td>4·5</td>
<td>Yellow-brown</td>
<td>0·12</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4·3</td>
<td>Pale yellow-brown</td>
<td>0·10</td>
</tr>
</tbody>
</table>
with apple PPO in the presence of excess (20 \textmu mol) ascorbic acid and Lineweaver-Burke plots of the initial oxidation rates gave the following $K_m$ values under these conditions: chlorogenic acid $1.66 \times 10^{-3} \text{M}$, D-catechin $1.48 \times 10^{-3} \text{M}$, caffeic acid $0.63 \times 10^{-3} \text{M}$.

(f) Inhibition of Apple PPO by Its Reaction Products

An experiment was devised (see Fig. 4 for details) to attempt to clarify the problem of the inhibition of PPO by the oxidation products of its substrate. The results demonstrate that enzyme inactivation occurred before all the substrate had been utilized, since a further addition of fresh enzyme (flask B) could catalyse the oxidation of the remaining substrate. By contrast extra substrate added to previously reacted enzyme (flask C) was not utilized.

![Graph](image)

Fig. 5.—Effect of cysteine on the oxidation of chlorogenic acid. Double-side-arm Warburg flasks contained 0.1 ml enzyme, 8 \textmu mol cysteine, 0.5 ml 1\% gelatine, and 0.1 ml phosphate-citrate buffer (pH 5.0). 12 \textmu mol chlorogenic acid added initially to flask A (control); 6 \textmu mol chlorogenic acid, followed by a further 6 \textmu mol from the second side-arm, to flask B.

(g) Action of Copper-complexing Reagents

Various workers have shown that PPO preparations contain copper as a prosthetic group (see review by Mason 1955). The effect of copper-complexing reagents on apple PPO was investigated by incubating the enzyme with 10 \textmu mol chlorogenic acid in the presence of the various reagents listed in Table I. It will be seen that diethylthiocarbamate and dimercaptopropanol were powerful inhibitors of the oxidation of chlorogenic acid.

(h) Effect of Thiol Compounds

Henze (1956) and Hulme (1958) have reported that cysteine and glutathione inhibited the browning of chlorogenic acid in the presence of PPO and they suggested that this action was due to the combination of sulphydryl groups with quinone
oxidation products of chlorogenic acid. These findings were further investigated in this laboratory by studying the effect of various levels of thiol compounds on the oxidation of chlorogenic acid and D-catechin by excess apple PPO. It was found that glutathione, L-cysteine, DL-homocysteine, cysteamine, and DL-penicillamine (β,β'-dimethylcysteine) behaved similarly in preventing browning if present at molar concentrations greater than that of chlorogenic acid and, as seen in Table 2, the final oxygen consumption was then less than one atom of oxygen per molecule chlorogenic acid. In contrast, less than equimolar quantities of these compounds did not inhibit browning and only slightly affected oxygen absorption. From Table 2 it will also be seen that other closely related compounds such as cystine, methionine, thioglycollic acid, and thiourea did not display these effects. Thiourea caused some inhibition of browning but without marked effect on oxygen absorption. It was therefore concluded that both -NH₂ and -SH groups were involved in these reactions.
It was visualized that formation of a colourless thiol–phenolic complex might not inactivate PPO as did the normal coloured oxidation products. An experiment was designed to test this hypothesis and the results (Fig. 5) show that the colourless cysteine–chlorogenic acid complex did not inactivate the enzyme.

Figure 6(a) shows the absorption spectra of the final reaction mixture of those reaction flasks which contained cysteine. The spectrum of the colourless reaction mixture was similar to that of chlorogenic or caffeic acid but without the characteristic point of inflexion, whereas the brown solution yielded a typical oxidized chlorogenic acid spectrum. Essentially similar spectra were given by mixtures containing glutathione or homocysteine. The behaviour of cysteine showed one minor difference in as much that equal molar ratios of chlorogenic acid and cysteine yielded a pinkish red colour after oxidation. This red complex may represent a half-way stage between the colourless and the brown complexes; its spectra had lost the trough at 273 m\(\mu\) but retained the peak at 318 m\(\mu\). Jurd (1956) has reported that the spectra of dihydroxyphenolic compounds undergo a characteristic bathochromic shift in the presence of excess borate. The solution of the colourless chlorogenic–glutathione complex was therefore saturated with sodium borate and the new spectrum recorded; it was found that the \(\lambda_{\text{max}}\) had shifted from 313 to 337 m\(\mu\), which suggested the presence of phenolic hydroxy groups in the complex.

The contents of the reaction flasks in these experiments were clarified by centrifugation and examined by paper chromatography using the butan-1-ol–acetic acid–water (40 : 10 : 22 v/v) solvent system. Those reaction mixtures which had browned gave rise to a diffuse slow-moving spot \((R_P = 0.14)\) whilst the colourless mixtures gave a definite but slow-moving spot \((R_P = 0.14)\) which exhibited a bright yellow fluorescence when examined under ultraviolet light, changing to a bright greenish yellow when exposed to ammonia fumes. Authentic chlorogenic acid had an \(R_P\) value of 0.63 under these conditions. When the chromatograms were tested with ninhydrin and heated the spots from both brown and colourless complexes gave purple colorations indicating the presence of amino groups. Other chromatograms were sprayed with the Hoepfner reagent \([5\% \ (w/v) \ \text{sodium nitrite plus 5\% \ (v/v) acetic acid}]\); the spots from the colourless complexes gave positive results, substantiating previous spectral evidence for the presence of phenolic hydroxyl groups in these compounds, whilst tests with sodium nitroprusside were negative, which suggested that the cysteine sulphydryl group was involved in complex formation.

Parallel experiments with mixtures of cysteine and \(\Delta\)-catechin yielded similar results to those obtained with cysteine–chlorogenic acid mixtures, i.e. no coloured oxidation products appeared when an excess of cysteine was present. Examination of the absorption spectra of the colourless cysteine–catechin complex \([\text{Fig. 6(b)}]\) shows a bathochromic shift from the catechin peak at 273 m\(\mu\) to a new maximum at 285 m\(\mu\).

IV. Discussion

The results presented in this paper lend further support to earlier suggestions that chlorogenic acid and catechin-type compounds are the major substrates for apple PPO and earlier work in these laboratories (Walker 1962) has indicated that sub-
strate concentration governs the degree of actual browning of any particular apple variety. Apple PPO can only utilize a limited range of phenolic compounds, being more specific in its action than the potato PPO investigated by Baruah and Swain (1959). The present results suggest that not only is the o-dihydroxyphenol structure essential for a compound to be readily oxidized by PPO but that other structural features play a part. For example the rate of oxidation of the following phenolic acids decreased in the order: hydrocaffeic acid > chlorogenic acid > isochlorogenic acid > caffeic acid > 3,4-dihydroxyphenylacetic acid > 3,4-dihydroxybenzoic acid.

Aesculetin was slowly oxidized but aesculin, quercetin, and its glycosides quercitrin and rutin did not react with apple PPO. Similarly Roberts and Wood (1951) also found that quercetin, its glycosides, and myricetin were less susceptible to oxidation by tea PPO than was catechin.

Modification of the catechol hydroxyl groups of these compounds rendered them immune to attack by PPO: for example, ferulic acid (4-hydroxy-3-methoxycinnamic acid) was not oxidized. This structural specificity has been elegantly demonstrated by Finkle and Nelson (1963) who treated caffeic and chlorogenic acids with the enzyme O-methyltransferase to yield ferulic and feruloylquinic acids respectively. Neither of these compounds was oxidized by a crude PPO preparation and these authors suggested a possible use for this system to control enzymic browning.

The PPO preparation used in the present work, as well as causing browning, exhibited some cresolase activity and slowly catalysed the hydroxylation of p-coumaric acid to caffeic acid. Hanson and Zucker (1963) have demonstrated the enzymatic hydroxylation of p-coumarylquinic acid to chlorogenic acid in potato tuber tissue, and it has been suggested that the normal in vivo metabolic role of these enzymes is that of "cresolases" for biosynthetic purposes (Mason 1956) whilst their "catecholase" activity would presumably be reserved as a defence mechanism.

Mason (1955) reported the reaction of catechol-type compounds with thiols, in the presence of excess PPO to yield pigmented or colourless compounds, excess glutathione causing the absorption of 1 atom of oxygen per molecule of catechol oxidized. Hense (1956) and Hulme (1958) later found that glutathione and cysteine could prevent the enzymic browning of solutions of chlorogenic acid. In the present work it was found that this effect was markedly dependent on the ratio of thiol to phenolic compound and thiol–chlorogenic acid ratios less than unity did not inhibit oxidation and concomitant brown pigment formation. Excess of thiol compound, however, prevented browning and resulted in a diminished oxygen absorption. Similar results were also observed for mixtures of cysteine and p-catechin. The results reported in this paper support the findings of Hulme in most respects except that it was found that when chlorogenic acid was incubated with excess glutathione and apple PPO, chromatography of the reaction mixture yielded only one spot. This compound, together with the colourless complexes formed by the reaction of chlorogenic acid with cysteine or homocysteine, were found to possess modified chlorogenic acid spectra, and reactive –NH₂ and –OH groups, whilst both –SH and –NH₂ groups appeared to be necessary for complex formation to occur. Since this reaction may be of importance for the maintenance of health in apple tissue, future work will be directed towards a study of its role in ripening fruit.
V. Acknowledgments

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VI. References

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