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EXTRACTION OF SOLUBLE LEAF ENZYMES WITH THIOLS AND OTHER REDUCING AGENTS

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Abstract—The activity of peptidase extracted from tobacco leaf was higher if thiols or other reducing agents were included in the extracting medium. The minimum concentration of the reducing agent (critical concentration) required in the extracting medium for maximum activity of peptidase was inversely correlated with the strength of the reducing agent. Chlorogenic acid inhibited peptidase activity in extracts prepared using the critical concentration of thioglycollate but not in extracts prepared with the critical concentration of metabisulphite. The critical concentration of metabisulphite was independent of concentration of enzyme but the critical concentration of thioglycollate increased with increasing concentration of enzyme. The mechanism whereby thiols and other reducing agents yield extracts with high peptidase activity is discussed in relation to inhibiting the synthesis of *o*-quinones and tannins from endogenous *o*-diphenols.

INTRODUCTION

ENDOGENOUS tannins, or quinones and tannins formed after cell rupture inhibit many enzymes extracted from plant tissue.[1-4] Since condensed tannins form readily from quinones,[1,5] preventing oxidation of phenols to quinones protects enzymes from inactivation during extraction from tissue rich in unpolymerized phenols. Since tobacco leaf contains an active *o*-diphenol: O_2 oxidoreductase (trivial name: *o*-diphenol oxidase)[6-10] with high affinity for chlorogenic acid, one of the predominant phenols found in tobacco leaf,[6,11,12] extracting active enzymes from the leaves requires efficient inactivation of this oxidase. Although some workers prefer removing phenols and tannins with polymers such as polyvinylpyrrolidone when extracting enzymes from plant tissue, others have shown that thiols effectively prevent inactivation.[4,13-16] We also have found that extracting with thioglycollate

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increased activity of peptidase and amino acyl-sRNA synthetase from tobacco leaf.[17,18] The work reported here shows that metabisulphite prevents inactivation even more efficiently and acts by inhibiting *o*-diphenol oxidase completely, while thioglycollate acts both by partially inhibiting *o*-diphenol oxidase and by reacting with quinones.

RESULTS

Throughout this paper, the critical concentration of a reducing agent is the minimum concentration in the extracting medium yielding enzyme with maximum activity. This is not always the minimum concentration inhibiting browning of the extract.

Effect of Chlorogenic Acid on Peptidase Activity of Extracts Prepared in Thioglycollate

If thioglycollate prevents inhibition of peptidase by inhibiting conversion of endogenous phenols to quinones and tannins, then adding chlorogenic acid to the assay should overcome the effect of thioglycollate more rapidly when the concentration of thioglycollate is low. Using peptidase prepared in 10 mM thioglycollate, incubating mixtures containing thioglycollate (5 or 10 mM) and chlorogenic acid (0–10 mM) were assayed after 1 and 5 hr and

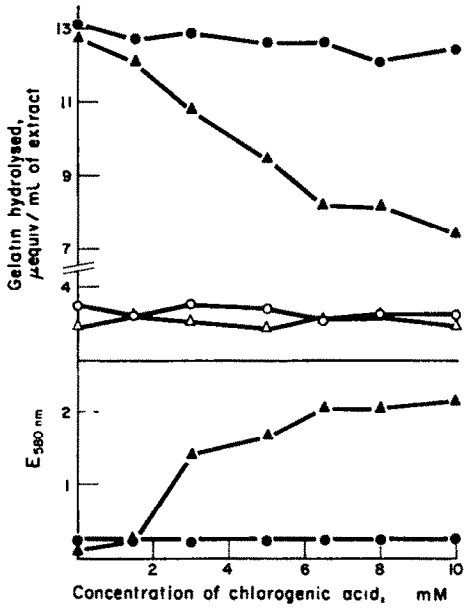

FIG. 1. EFFECT OF CONCENTRATION OF CHLOROGENIC ACID ON PEPTIDASE ACTIVITY AND BROWN PIGMENT (E_{580}) FORMED AFTER 1 AND 5 HR.

Gelatin hydrolysed after 1 hr in reaction mixtures containing 5 mM (△) and 10 mM (○) thioglycollate. Gelatin hydrolysed after 5 hr in reaction mixtures containing 5 mM (▲) and 10 mM (●) thioglycollate. Brown pigment (E_{580}) formed after 5 hr in reaction mixtures containing 5 mM (▲) and 10 mM (●) thioglycollate. The enzyme was prepared from tobacco leaf by extracting with buffer containing thioglycollate (10 mM). Reaction mixtures contained 0·50 ml enzyme extract (containing 5·0 μmoles thioglycollate), 1·65 to 11·0 μmoles chlorogenic acid or water, 5·5 μmoles additional thioglycollate or water, and 20 mg gelatin in a total volume of 1·10 ml.

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browning after 5 hr only. After 5 hr, chlorogenic acid induced formation of a brown pigment and inhibited peptidase activity in incubating mixtures containing 5 mM thioglycollate (Fig. 1). Chlorogenic acid neither induced browning nor inhibited peptidase during the first hour of incubation. Thus increasing concentration of chlorogenic acid and decreasing concentration of thioglycollate accelerated inhibition of peptidase, though only after a lag phase of at least 1 hr.

An extract prepared in buffer containing thioglycollate (10 mM) was assayed for peptidase activity and brown pigment with and without chlorogenic acid (10 mM) (Fig. 2). Peptidase activity in reaction mixtures without chlorogenic acid did not decrease until 210 min but with chlorogenic acid decreased after 30 min. In both reaction mixtures, browning occurred 90

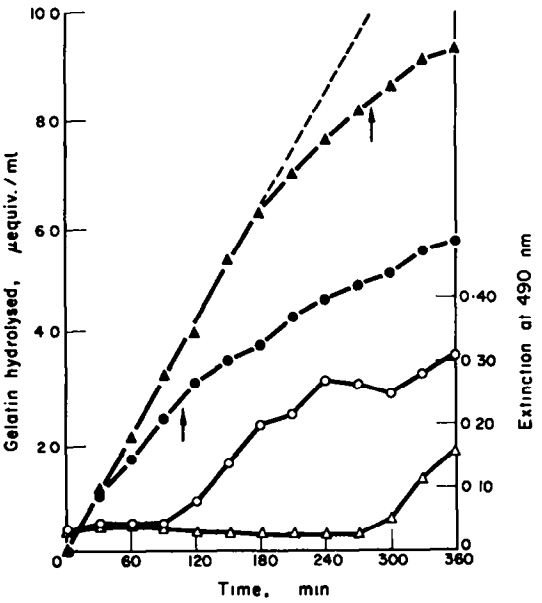

FIG. 2. EFFECT OF CHLOROGENIC ACID ON ACTIVITY OF PEPTIDASE AND BROWN PIGMENTATION.
Time course of hydrolysis of gelatin with (●) and without (▲) chlorogenic acid (10 mM) and of brown pigmentation (E_{490}) with (○) and without (△) chlorogenic acid (10 mM). Arrows indicate the onset of browning. The enzyme was prepared from tobacco leaf by extracting with buffer containing thioglycollate (10 mM). Reaction mixtures contained 3·0 ml extract (containing 30 μmoles thioglycollate), 60 μmoles chlorogenic acid or water, and gelatin (120 mg) in a final volume of 6·3 ml.

min after the decrease in activity of peptidase. This suggests that the colourless inhibitor is a precursor of the brown pigment which is also an inhibitor.

Effect of Including Chlorogenic Acid in Extracting Medium Containing 10 mM Thioglycollate

When extracting peptidase from tobacco leaf without using reducing agents, the brown pigment appears after grinding tissue for 3 min at 1°. When chlorogenic acid was added to extracts prepared in 10 mM thioglycollate (Fig. 1) the brown pigment formed slowly. McClendon[19] reported that tobacco *o*-diphenol oxidase was particulate as well as soluble. Since adding chlorogenic acid (20 mM) to the extracting medium containing thioglycollate (10 mM) did not reduce peptidase activity or induce browning, we conclude that loss of a

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particulate oxidase during extraction does not lower the rate of synthesis of the inhibitor or the brown pigment.

Effect of Including Thiols and Other Reducing Agents in the Extracting Medium on Activity of Peptidase and Formation of Brown Pigment

Extracts prepared in media containing dithionite (10 mM) and metabisulphite were colourless and the peptidase activity was greater than in extracts prepared using thioglycollate (10 mM) (Table 1). Since neither dithionite not metabisulphite are thiols, we conclude that the reducing power of thioglycollate prevents the inhibition of peptidase. The less powerful reducing agents (10 mM sodium glycollate, 10 mM sodium thiosulphate) did not prevent inhibition of peptidase although thiosulphate prevented browning. Activity of extracts prepared in media containing L-cysteine (10 mM) was similar to that in extracts prepared in thioglycollate (10 mM).

TABLE 1. EFFECT OF INCLUDING REDUCING AGENTS IN THE EXTRACTING MEDIUM ON PEPTIDASE ACTIVITY AND BROWN PIGMENTATION

Addition to extracting medium (Conc. 10 mM)	Brown pigmentation after incubation (E_{490})	Peptidase activity (units/ml)
—	0·241	7·7
Sodium thioglycollate	0·036	41·0
Sodium glycollate	0·247	8·5
Sodium thiosulphate	0·085	9·2
Sodium dithionite	0·032	52·2
Potassium metabisulphite	0·032	53·0

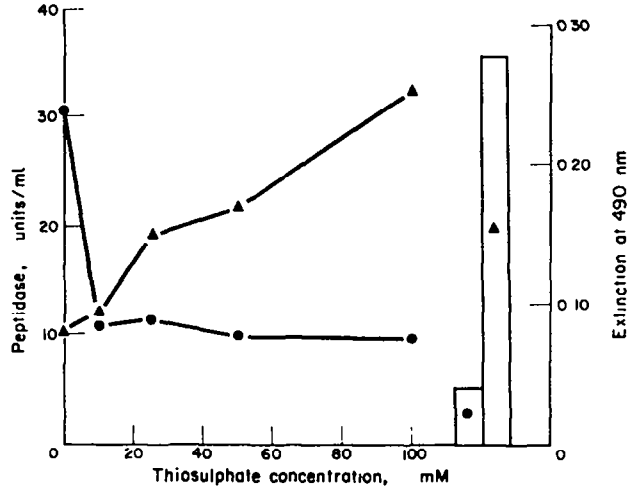

FIG. 3. EFFECT OF SODIUM THIOSULPHATE IN THE EXTRACTING MEDIUM.

Peptidase activity (▲) and brown pigment (E_{490}) (●) in extracts from tobacco leaf. The histograms show activity and pigment in a comparable extract prepared in thioglycollate (10 mM). All concentrations in the reaction mixtures were half the concentration in the extracting media.

Extracts prepared in 10 mM thiosulphate remained colourless during assay but peptidase activity was as low as in control extracts (Table 1). Extracts prepared in ascorbic acid (10 mM) were colourless, but turned brown during assay and peptidase activity was low. The following experiment demonstrated the independence of the brown pigment and the peptidase inhibitor. Extracts were prepared from leaf disks with extracting medium containing 10–100 mM sodium thiosulphate and the brown pigment and peptidase were measured after 1 hr (Fig. 3). The activity of peptidase increased with increasing concentration of thiosulphate while 10 mM thiosulphate inhibited formation of the brown pigment completely. A high concentration of thiosulphate (100 mM), a weak reducing agent, simulates 10 mM thioglycollate, confirming that the effect of thioglycollate is a function of its reducing power. On the other hand, metabisulphite, a stronger reducing agent than thiosulphate, has a low critical concentration (3·3 mM) (Fig. 4).

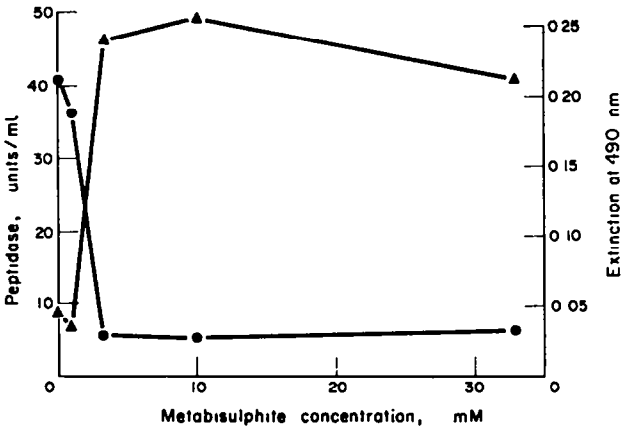

FIG. 4. EFFECT OF POTASSIUM METABISULPHITE IN THE EXTRACTING MEDIUM.

Peptidase activity (▲) and brown pigment (E_{490}) (●) in extracts from tobacco leaf. All concentrations in the reaction mixtures were half the concentrations in the extracting media.

Effect of Adding Chlorogenic Acid to an Assay of an Extract Prepared with Metabisulphite in the Extracting Medium

The peptidase activity of extracts prepared in media containing thioglycollate (10 mM) decreased with increasing concentration of chlorogenic acid when assayed after 5 hr (Fig. 1). On the other hand, when an extract prepared at the critical concentration of metabisulphite (4 mM) was assayed for peptidase activity after 1 and 5 hr, adding chlorogenic acid (2–10 mM) to the incubating mixture did not decrease peptidase activity after 1 or 5 hr or induce browning after 24 hr.

Oxygen Uptake of Incubating Mixtures Containing Extracts Prepared from Tobacco Leaf in Thioglycollate and Metabisulphite and the Effect of Chlorogenic Acid on Oxygen Uptake

Extracts were prepared in thioglycollate (12·7 mM) and metabisulphite (4 mM) and peptidase reaction mixtures were prepared in Warburg manometer flasks with and without chlorogenic acid (10 mM). Oxygen uptake was measured manometrically at 40°. Adding chlorogenic acid to the extract prepared in thioglycollate increased the initial rate of oxygen uptake; the rate increased sharply at about 9 hr and the mixture turned brown (Fig. 5). In the extract

without chlorogenic acid the sharp increase in rate and brown pigment did not occur until 20 hr. Chlorogenic acid did not alter the rate of oxygen uptake or induce browning in extracts prepared in metabisulphite and the rate of uptake was below that of extracts prepared in thioglycollate (Fig. 5).

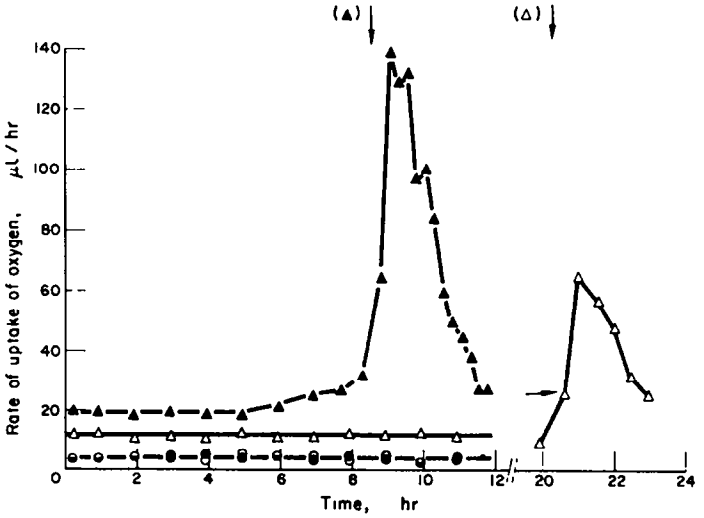

FIG. 5. CHANGE IN RATE OF UPTAKE OF OXYGEN BY EXTRACTS FROM TOBACCO LEAF PREPARED IN POTASSIUM METABISULPHITE OR SODIUM THIOGLYCOLLATE INCUBATED WITH OR WITHOUT CHLOROGENIC ACID.

Extracts prepared in metabisulphite (4 mM) and assayed with (●) and without (○) chlorogenic acid (10 mM). Extracts prepared in thioglycollate (12·7 mM) and assayed with (▲) and without (△) chlorogenic acid (10 mM). Arrows indicate the onset of browning. Reaction mixtures contained 2·0 ml extract (containing 25·4 μmoles thioglycollate or 8 μmoles metabisulphite), 40 μmoles chlorogenic acid or water and gelatin (80 mg) in a final volume of 4·2 ml. Each point is the mean of duplicate determinations. Temperature: 40°.

Peptidase Activity of Extracts Prepared without Thioglycollate Followed by Re-extraction from the Residue with Thioglycollate

Rowsell and Goad[20, 21] found that the latent β-amylase of wheat endosperm bound to glutenin was released by treating the endosperm with thiols, sodium sulphite or proteolytic enzymes. They concluded that the enzyme was bound by a disulphide bond and that thiols or sodium sulphite competed for the SH groups of glutenin, displacing β-amylase. A similar process could explain the increased peptidase activity extracted from tobacco leaf when thioglycollate is included in the extracting medium.

If this hypothesis is correct, peptidase activity equal to the difference in activity between preparations extracted with and without thioglycollate should be recovered by re-extracting the residue remaining after an initial extraction without thioglycollate, in buffer containing thioglycollate. Samples of thirty leaf disks were ground in 3·0 ml buffer, with and without thioglycollate (30 mM). The supernatant solutions were recovered by centrifugation (15,000 *g* for 45 min) and retained for assay (first extract). The precipitates were ground again with 3·0 ml buffer, with or without thioglycollate (30 mM), and the supernatant solutions recovered by centrifugation (second extract). All extracts were assayed for peptidase activity (Table 2).

[20] E. V. ROWSELL and L. J. GOAD, *Biochem. J.* **84**, 73P (1962a).
[21] E. V. ROWSELL and L. J. GOAD, *Biochem. J.* **84**, 73P (1962b).

TABLE 2. PEPTIDASE ACTIVITY OF EXTRACTS PREPARED WITH AND WITHOUT THIOGLYCOLLATE, AND PEPTIDASE ACTIVITY OF EXTRACTS AFTER RE-EXTRACTION OF THE RESIDUES WITH AND WITHOUT THIOGLYCOLLATE

Treatment	First extraction		Second extraction	
	Conc. of thioglycollate (mM)	Peptidase* (units/ml)	Conc. of thioglycollate (mM)	Peptidase* (units/ml)
1	0	11·8	0	5·0
2	0	10·1	30	3·9
3	30	43·7	30	4·7

* Activities shown are mean values from duplicate extractions.

Adding thioglycollate to the medium used in preparing the second extract did not increase the peptidase activity of the extract. Activity in either of the second extracts prepared with thioglycollate did not approach the difference in activity found in first extracts prepared with and without thioglycollate (approx. 30 units/ml). Thus we conclude that thiols do not compete with peptidase for binding sites on proteins in the residue remaining after extraction. Further, the relation between the activity of peptidase and concentration of thioglycollate in the extracting medium is sigmoidal[17] (Fig. 6), not the hyperbolic relation expected if the effect of the thioglycollate was due to competition.

Effect of the Physiological Condition of Tobacco-leaf Tissue on the Critical Concentration of Thioglycollate and Metabisulphite

Anderson and Rowan[17] reported that the critical concentration of thioglycollate for extracting peptidase from tobacco-leaf tissue (extracted at 10 disks/ml) was 10 mM. However, in the course of the work reported here, the critical concentration varied between 0·5 and 10 mM. The critical concentration was higher with plants grown outside in the garden than with plants grown in pots in the glass-house, and was higher with mature plants than with immature plants. By contrast, the critical concentration of metabisulphite for tobacco-leaf tissue in any kind of physiological condition was invariably between 3 and 4 mM.

Effect of the Amount of Tissue Extracted/Unit Volume of Extracting Medium on the Critical Concentration of Thioglycollate and Metabisulphite

Extracts were prepared from matched samples of 10 and 20 disks with 2·0 ml extracting medium containing 0·5–7·5 mM sodium thioglycollate or 1·0–8·0 mM potassium metabisulphite. The extracts were dialysed for 18 hr against 30 ml of the appropriate extracting medium with one change of medium, and assayed for peptidase. Brown pigment was measured by diluting 0·4 ml incubating mixture with 3·0 ml distilled water, recovering the supernatant solution by centrifugation and reading the extinction at 490 nm.

The critical concentration of metabisulphite was 4 mM, irrespective of whether the disks were extracted at 5 or 10 disks/ml. This concentration of metabisulphite completely inhibited the formation of the brown pigment at both 5 and 10 disks/ml. The critical concentration of thioglycollate for disks extracted at 5 disks/ml was 2·5 mM but was 5·0 mM for disks extracted at 10 disks/ml (Fig. 6).

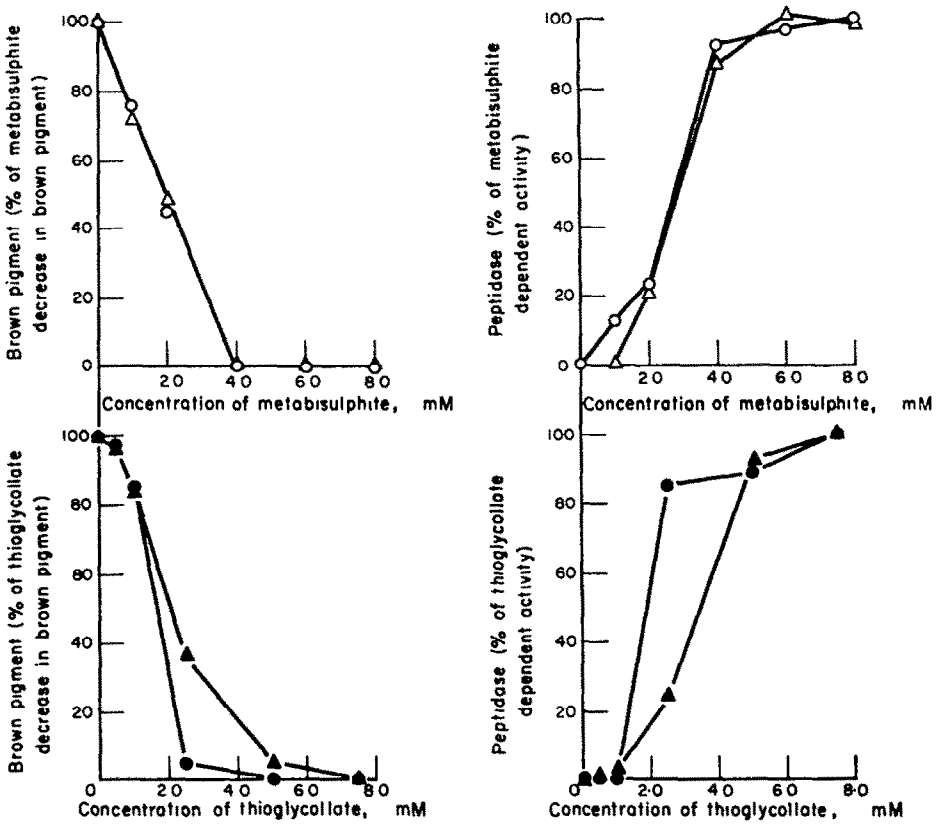

FIG. 6. EFFECTS OF CONCENTRATION OF SODIUM THIOGLYCOLLATE AND POTASSIUM METABISULPHITE IN THE EXTRACTING MEDIUM ON PEPTIDASE ACTIVITY AND BROWN PIGMENT (E_{490}) FORMED IN EXTRACTS PREPARED USING 5 OR 10 DISKS OF TOBACCO LEAF/ML OF EXTRACTING MEDIUM.

Extract concentrations: ○—5 disks/ml extracting medium containing metabisulphite; △—10 disks/ml extracting medium containing metabisulphite; ●—5 disks/ml extracting medium containing thioglycollate; ▲—10 disks/ml extracting medium containing thioglycollate.

Left: Brown pigment as a percentage of the total decrease in E_{490} due to metabisulphite or thioglycollate. Right: Peptidase activity as a percentage of maximum increase in activity observed using metabisulphite or thioglycollate in the extracting medium.

DISCUSSION

Since inhibition of peptidase in extracts unprotected by reducing agents precedes formation of brown pigment, the inhibitor is colourless (Figs. 2 and 3; Table 1). Reducing agents prevent inhibition of peptidase and pigment formation at concentrations correlated with reducing power (Figs. 3 and 4) and we conclude that the primary effect of thioglycollate is to lower the electrode potential of the extracting medium.

Since extracts prepared in thioglycollate (10 mM) begin losing activity after approx. 3 hr (Fig. 2) and eventually turn brown, we conclude that thioglycollate slowly disappears from the incubations, allowing accumulation of the peptidase inhibitor followed by formation of tannin. Oxidation of chlorogenic acid is not completely suppressed by thioglycollate (Fig. 5) and we presume that the quinone is formed.[10]

When extracts prepared with thioglycollate (10 mM) were incubated with chlorogenic acid (Fig. 5) oxygen uptake increased rapidly after approx. 9 hr; by this time oxygen uptake in the incubation without chlorogenic acid was 4·0 μmoles. Assuming that thioglycollate is oxidized to dithioglycollate either enzymically[22] or non-enzymically[23] then the oxygen uptake after 9 hr is equivalent to only 16·0 μmoles of the 25·4 μmoles thioglycollate originally present.

We conclude that quinones formed by oxidation of chlorogenic acid remove the remaining thioglycollate either by oxidation to the disulphide or by forming condensation compounds.[1] The uptake of oxygen in the thioglycollate extract incubated with chlorogenic acid (Fig. 5) is 8·9 μmoles in 9 hr. Oxidation of 16·0 μmoles of thioglycollate accounts already for 4·0 μmoles; the remaining 4·9 μmoles is equivalent to 9·8 μmoles quinone which would react with 9·8 μmoles thioglycollate. Thus the total thioglycollate removed by direct oxidation and reaction with quinones is 25·8 μmoles, agreeing closely with the amount of thioglycollate in the incubating mixture (25·4 μmoles). The rapid uptake of oxygen after 9 hr (Fig. 5) suggests that thioglycollate had inhibited *o*-diphenol oxidase until this time, though Pierpoint[10] believed that thioglycollate acted entirely by reducing quinones.

Since rate of removal of thioglycollate is a function of concentration of quinone and activity of thiol oxidase, the critical concentration of thioglycollate will vary between extracts (Fig. 6). On the other hand, the critical concentration of metabisulphite, acting directly on *o*-diphenol oxidase, remains constant. Variation in critical concentration with physiological condition of the tobacco-leaf tissue reported here is consistent with variation in concentration of chlorogenic acid with environment[24] and with variations in concentration of other phenols.[11]

Although browning of extracts often accompanies low activity of peptidase, activity was low in clear extracts prepared in thiosulphate (10–50 mM) (Fig. 3). Since adding thioglycollate to an extract prepared without thioglycollate does not restore activity of peptidase to the level in extracts prepared in thioglycollate,[17] the inhibition does not consist entirely of oxidizing SH groups of proteins to S—S groups, for this effect should be fully reversible. Also, thioglycollate added to the extracting medium does not act by preventing SH groups of peptidase from forming S—S bonds with protein insoluble in the extracting medium (Table 2).

Although polymers are effective in protecting enzymes during extraction from a number of tissues,[3,25–29] polyvinylpyrrolidone did not protect efficiently mitochondria isolated from white potato tuber.[29] Using metabisulphite in the extracting medium, Stokes and Anderson (unpublished) have prepared tightly coupled mitochondria from potato and Chalmers and Rowan (unpublished) have prepared an active phosphofructokinase from fruit of tomato in this laboratory. Working at University College London, Anderson (unpublished) has obtained a 2–3-fold increase in activity of amino acyl-sRNA synthetases from the plumule of seedlings of *Phaseolus vulgaris* by including metabisulphite or thioglycollate in the extracting medium. Thus, strong reducing agents (e.g. metabisulphite or dithionite) seem a convenient alternative to adding polymers during enzyme extraction, are more effective than thiols and are effective with tissue such as potato tuber where polyvinylpyrrolidone is relatively ineffective.

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EXPERIMENTAL

Chlorogenic acid (C grade) was supplied by Calbiochem, Los Angeles 63, Calif., U.S.A.

Peptidase was extracted from tobacco-leaf disks in phosphate-citrate buffer (pH 5·2) and assayed by the method of Anderson and Rowan[17] except that thioglycollate was replaced in the extracting medium by other reagents when required. Control extracts were prepared using buffer without reagents added. Unless specified otherwise peptidase was extracted at 10 disks/ml extracting medium and the activity determined after digestion for 1 hr at 40° at pH 5·2. The mass of each leaf disk was approx 30 mg.

Brown pigment in the incubating mixtures prepared without reducing agents absorbs light between 420 and 720 nm without showing a definite E_{max} while mixtures prepared with reducing agents absorb little light above 480 nm. Accordingly, brown pigment was measured by removing 0·2 ml of incubating mixture, diluting with 3·0 ml distilled water and reading extinction immediately in the spectrophotometer (Unicem SP 500) at 490 nm[30] or 580 nm.

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