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Biosynthesis of Ethylene

ENZYMES INVOLVED IN ITS FORMATION FROM METHIONAL

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1. Two enzymes were shown to be necessary for the production of ethylene from methional; they were separated from extracts of cauliflower florets by fractionation on Sephadex and other methods. 2. The first enzyme, generating hydrogen peroxide, appears to be similar to the fungal glucose oxidase, for like the latter it is highly specific for its substrate D-glucose. 3. The second enzyme, in the presence of cofactors and peroxide generated by the first enzyme, cleaves methional to ethylene. 4. It was also found that hydrogen peroxide in these reactions may be replaced by hydroperoxide generated from linolenic acid by lipoxidase enzymes. 5. Dihydroxyphenols were shown to have a marked inhibitory effect on these reactions and to account for the initial phase of low activity that is always observed in aqueous extracts prepared from the floret tissue.

The enzymic formation of ethylene from methional in extracts prepared from the florets of cauliflowers has been described by Mapson & Wardale (1967). In these studies evidence was obtained that two separate enzymes were involved, the first generating peroxide under aerobic conditions (oxidase activity), the second using this peroxide to catalyse the breakdown of methional to ethylene (methional-cleaving enzyme). In the production of ethylene by this system a phase of low activity always preceded one of rapid activity. The duration of the first phase was found to vary from 1 hr. to as long as 24 hr. in extracts prepared from cauliflowers at different seasons of the year. The present paper describes the separation of the two enzymes and the results of experiments designed to investigate the reason for the initial phase of low activity.

EXPERIMENTAL

Cauliflowers. These were purchased from the local market and used the same day. A supply was also obtained from the National Institute of Agricultural Botany, Cambridge, and used immediately after cutting.

Enzymes and chemicals. Fungal glucose oxidase (30 Sigma units/mg.) and catalase (19000 Sigma units/0·1 ml.) were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A., and lipoxidase (50000 units/mg.) was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks. The methyl esters of linoleic acid, linolenic acid and oleic acid were obtained from the Hormel Institute, Austin, Minn., U.S.A., and polyamide (Woelm T.L.C.) from M. Woelm, Eschwege, Germany. 3-Methylthiopropan-1-ol was prepared from methional by reduction with $NaBH_4$, and 3-methylthiopropionic acid was prepared from sodium methanethiol and the ethyl ester of 3-chloropropionic acid. The methyl ester of 3-methylthiopropionic acid was purchased from Koch–Light Laboratories Ltd. Methional was prepared from acrolein and methanethiol as described by Mapson & Wardale (1967).

Cofactor. In most of the experiments the cofactor was supplied either as a freshly prepared aqueous extract from cauliflower florets heated at 100° and centrifuged to remove denatured proteins (extract *X*), or as the aqueous extract that had first been exposed at room temperature to O_2 for 24 hr. before heating and removal of protein (extract *Y*). It was added to enzyme digests in amounts obtained from 10–15 g. of floret tissue. In experiments during the purification of the methional-cleaving enzyme and in experiments where glucose-free extracts were required, the cofactor was supplied as *p*-hydroxybenzoic acid (1 mg.) plus 0·2 ml. of a fraction isolated from extracts of floret tissue (extract *Z*). This fraction contained mainly the organic acids extracted from an acid aqueous extract with diethyl ether.

Enzyme system for conversion of methional into ethylene and the determination of ethylene. These were as described by Mapson & Wardale (1967). The unit of activity of the methional-cleaving enzyme was defined as the amount of enzyme that will produce 1 μmole of ethylene in 30 min. at pH 6·8 and 25° in a 50 ml. flask with 5 μg. of fungal glucose oxidase ($\equiv O_2$ uptake of 55 μl./hr.) in a total volume of 10 ml.

Determination of oxidase. This was carried out essentially by the method of Keilin & Hartree (1948). Uptake of O_2 was measured manometrically in a Warburg apparatus at 25°. The reaction mixture contained enzyme solution, 0·1 M-sodium phosphate buffer, pH 6·8, and 0·1 ml. of catalase solution in a volume of 3 ml. At zero time 0·2 ml.

of 20% (w/v) glucose solution was tipped from the side arm. A blank solution without glucose was run as control. The unit of enzyme activity was defined as the amount of enzyme that will oxidize 1 μmole of glucose to gluconic acid and hydrogen peroxide/hr. at pH 6·8 in the presence of catalase ($\equiv O_2$ uptake of 11·2 μl./hr.).

Determination of protein. The protein content of the various enzyme preparations was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Gel filtration. The Sephadex column (2·5 cm. × 45 cm.) was first washed with 0·1 M-sodium phosphate buffer, pH 6·8. The enzyme was concentrated to 10–15 ml. ($\equiv$250 g. of original florets) by filtration under reduced pressure through collodion-shell-membrane filters, and then added to the column. Elution was with the sodium phosphate buffer, fractions being collected after the void volume had passed. The first few fractions were always cloudy.

Preparation of the methional-cleaving enzyme

Preparation of extract. The florets (600 g.) of cauliflower, after separation from leaves and stalk, were extracted with 600 ml. of a chilled sucrose–phosphate solution (0·4 M-sucrose–0·1 M-sodium phosphate buffer, pH 7·2) by maceration in a Waring Blendor. The particulate elements were then removed by centrifugation at 20000*g* for 20 min. The supernatant solution was collected and fractionated with $(NH_4)_2SO_4$. Tests showed that the proteins precipitated in the range between 17·6 g. and 39·0 g. of $(NH_4)_2SO_4$/100 ml. (30–60% saturation) contained enzymes capable of producing ethylene from methional. This fraction, after dialysis against 0·1 M-sodium phosphate buffer, pH 6·8, to remove diffusible components, was centrifuged at 20000*g* for 30 min. to clarify the solution. Further purification could be achieved by acidifying the chilled solution to pH 4·0 with 3 N-HCl, removing inert material by centrifugation and adjusting again to pH 6·8 with 3 N-NaOH.

Fractionation with Sephadex G-25. The extract was further fractionated by the addition of 10 g. of dry Sephadex G-25 (coarse grade)/100 ml. of extract. After 10 min. the gel grains were removed from the mixture by centrifugation and the solution was concentrated by adding solid $(NH_4)_2SO_4$ (47·2 g./100 ml. of solution) to give 70% saturation. The precipitate was collected, dissolved in 15 ml. of 0·01 M-phosphate buffer, pH 7·4, and dialysed for 2 hr. against 1 l. of the same buffer.

Fractionation with DEAE-cellulose. Before use the DEAE-cellulose was treated with 1 N-NaOH and centrifuged. It was then washed free of NaOH with water, suspended in 0·01 M-phosphate buffer, pH 7·4, and centrifuged, the process being repeated several times. The purified DEAE-cellulose was poured into a chromatography column (2·5 cm. × 45 cm.) as a thick slurry and allowed to pack down. The enzyme solution (15 ml.) was then added to the column without pressure and the column washed with 0·01 M-phosphate buffer, pH 7·4. The first 50 ml. of solution was collected when protein appeared from the column; this fraction contained the methional-cleaving enzyme and the oxidase enzyme. It was concentrated to 8 ml. in a collodion-membrane filter and dialysed against 0·005 M-phosphate buffer, pH 6·8, for several hours.

Adsorption on CM-Sephadex C-50. The cation-exchanger was allowed to swell in a large excess of 0·05 M-phosphate buffer, pH 6·8, and the supernatant liquid was replaced several times by fresh buffer solution over a period of 24 hr. The equilibrated ion-exchanger was then packed in a chromatography column (1·5 cm. × 30 cm.) and the enzyme solution applied to the top of the bed. The column was well washed with 0·05 M-phosphate buffer, pH 6·8, until all protein not adsorbed had been removed. The adsorbed enzymes were then eluted with 0·05 M-phosphate buffer, pH 6·8, containing progressively increasing amounts of NaCl. The salt-gradient buffer was prepared by dripping 0·3 M-NaCl in 0·05 M-phosphate buffer, pH 6·8, into 150 ml. of the phosphate buffer alone and continuously feeding the overflow to the column. Samples (10 ml.) were collected from the column, and those containing the methional-cleaving enzyme were combined and concentrated in a collodion-membrane filter and then kept at 1°; no loss of activity was observed over a period of several months. During this stage of purification of the methional-cleaving enzyme the oxidase enzyme was not recovered. The purification achieved by these operations is summarized in Table 1.

RESULTS

Separation of the peroxide-generating enzyme from the methional-cleaving enzyme

The suggestion that a peroxide-generating enzyme was involved in the production of ethylene from methional was based on the following observations (Mapson & Wardale, 1967): (1) fungal glucose oxidase, when added to extracts, caused a rapid production of ethylene; (2) the addition of catalase extended indefinitely the phase of low activity; (3) the addition of hydrogen peroxide could achieve the same result as the addition of glucose oxidase. Attempts were therefore made to obtain evidence for or against the presence of an oxidase identical with or similar to glucose oxidase.

Separation by gel filtration. An extract containing both the methional-cleaving enzyme and the oxidase enzyme was prepared as described in the Experimental section as far as acidification to pH 4·0, removal of inert material by centrifugation and neutralization again to pH 6·8. This extract was further fractionated by filtration on columns containing Sephadex G-100, G-150 and G-200, which resolve components of molecular weight in the ranges 4000–150000, 5000–400000 and 5000–800000 respectively. Isolation of protein fractions having methional-cleaving activities, when supplemented with exogenous glucose oxidase, indicated K_d values of 0·15, 0·37 and 0·46 from the respective columns, suggesting that the molecular weight of this enzymic protein was about 150000.

In an early experiment four protein fractions, *A*, *B*, *C* and *D*, were isolated after filtration on Sephadex G-150. The activity of the oxidase, as determined by the glucose oxidase test, was confined mainly to fraction *A*, though a small amount of activity was also present in fraction *B* (oxygen uptake 3 μl./mg. of protein/hr. in fraction *A* compared with 0·9 μl./mg. of protein/hr. in fraction

Table 1. *Stages in the purification of the methional-cleaving enzyme*

Stage of purification	Methional-cleaving enzyme (units/kg. of florets)	Specific activity (units/mg. of protein)	Total protein (mg./kg. of florets)	Oxidase enzyme (units/kg. of florets)
Non-particulate fraction in 0·1 M-phosphate buffer, pH 7·2	155	0·03	4770	—
30–60% saturation with $(NH_4)_2SO_4$ and dialysis	125	0·11	1160	35·6
Acidification to pH 4·0	97	0·18	545	35·2
Fractionation with Sephadex G-25 (coarse grade)	88·6	0·21	430	35
Passage through DEAE-cellulose	54·7	0·63	87	24·2
Adsorption on CM-Sephadex C-50 and gradient elution	17	9	1·9	Nil

B). The activity of the methional-cleaving enzyme, however, was found mainly in fraction *B*.

In general, Sephadex G-200 was used for gel filtration, but it was not completely effective in separating the oxidase enzyme from the methional-cleaving enzyme. The former was always eluted just in front of the latter.

Separation by uranyl acetate. The material used for this step was extracted as before, but the ammonium sulphate fraction (30–60% saturation) was taken up in 0·01 M-sodium acetate buffer, pH 5·0, and dialysed against 1 l. of the same buffer. The extract was acidified to pH 4·0 and, after removal of inert material, the pH was readjusted to 4·5. The extract was then treated with 4 ml. of 1% uranyl acetate/100 ml. of extract and left at 10°. After 30 min. the precipitate was collected by centrifugation and the clear supernatant was returned to 10°. The fall in activity of the methional-cleaving enzyme was noted over a 30 hr. period, and when all activity had disappeared the solution was concentrated by adding solid ammonium sulphate (47·2 g./100 ml. of solution) to give 70% saturation. The precipitate was taken up in 0·01 M-acetate buffer, pH 5·5, and dialysed for 4 hr. against the same buffer. This extract had no methional-cleaving enzyme present, but did contain oxidase activity when measured by oxygen uptake.

Some oxidase activity was also recovered from the precipitate formed by the uranyl acetate after the precipitate had been washed with water and the enzyme extracted with 0·2 M-phosphate buffer, pH 6·8. This was the method used for the purification of glucose oxidase in mould cultures (Bentley, 1957), but in floret tissue less than 10% of the oxidase was precipitated by uranyl acetate, the remainder being recovered from the supernatant.

Substrate specificity of oxidase

The specificity of the plant oxidase towards various pentose and hexose sugars was examined. Only D-glucose was found to be an effective substrate for the enzyme. These results were then compared with those in which the ability of the sugars to promote the synthesis of ethylene was tested. For this purpose the sugar was added to a well-dialysed enzyme fraction containing oxidase and methional-cleaving enzyme with the addition of an extract containing the cofactor, either one that had previously been treated with glucose oxidase to remove D-glucose and then heated to inactivate the added oxidase, or one in which the sugars had been removed by chemical fractionation (extract *Z*). The results showed that the ability of a sugar to promote the uptake of oxygen in the glucose oxidase test paralleled its ability to catalyse the formation of ethylene from methional. It is noteworthy that the specificity of the plant oxidase appeared to be as high as that of the fungal enzyme, for 2-deoxy-D-glucose was the only substrate tested, other than D-glucose, that showed any sign of acting as a substrate. Even with this substrate the rate at which the ethylene reaction was stimulated and the uptake of oxygen in the glucose oxidase test were only about 10% of values observed with D-glucose. In this respect the plant enzyme utilized 2-deoxy-D-glucose to a smaller extent than the fungal enzyme, where the rate relative to glucose was 25% (Dixon & Webb, 1966).

Peroxide and ethylene formation

The presence of a peroxide-generating enzyme with the methional-cleaving enzyme is required for the formation of ethylene from methional, as shown in Fig. 1. In this experiment the activities of protein fractions *A* and *B* isolated after filtration through Sephadex G-150 were tested for their ability to generate ethylene in the presence of the heat-stable cofactor (extract *Y*). The enzyme fractions separately produced either no ethylene (fraction *B*) or only a small amount (fraction *A*), but readily produced it when both were present.

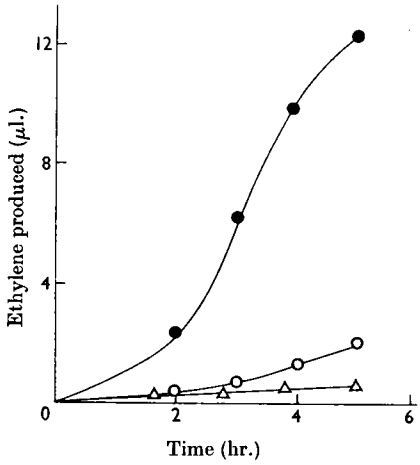

Fig. 1. Involvement of two enzymes (the methional-cleaving enzyme and peroxide-generating enzyme) in the production of ethylene from methional. The two enzyme fractions were separated on Sephadex G-150 by elution with 0·1 M-sodium phosphate buffer, pH 6·8. Flasks contained EDTA (2 mM), glucose (1%, w/v), cofactor (extract *Y*) and methional (1 mM) in 0·1 M-sodium phosphate buffer, pH 6·8, in a total volume of 10 ml. ○, Fraction *A*; △, fraction *B*; ●, both fractions.

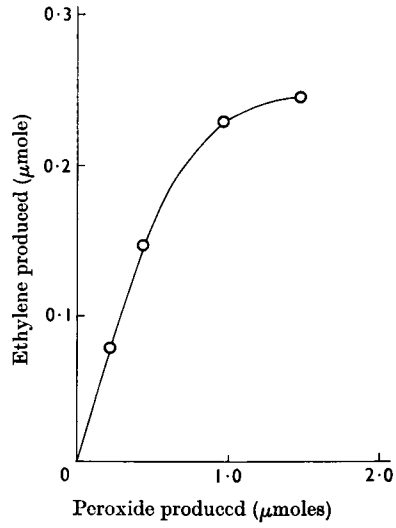

Fig. 2. Relationship between the production of ethylene and formation of peroxide. The methional-cleaving enzyme fraction was eluted from Sephadex G-200 and contained only a small amount of oxidase activity. The small amount of ethylene generated by this oxidase was subtracted as a blank value from that formed in the presence of the added fungal oxidase. Flasks contained the enzyme fraction (1·5 units), EDTA (2 mM), glucose (1%, w/v), cofactor (extract *Y*) and methional (1 mM) in 0·1 M-sodium phosphate buffer, pH 6·8, in a total volume of 10 ml. at 25°. Fungal glucose oxidase in various concentrations was added, and the rate of ethylene production was measured. The oxygen uptake due to the fungal glucose oxidase added was determined in each case under the same conditions of buffer and temperature.

Since fraction *A* contained only small amounts of the methional-cleaving enzyme but the main portion of the peroxide-generating enzyme, whereas fraction *B* contained mainly the methional-cleaving enzyme and only a small amount of the peroxide-generating enzyme, the fact that greater activity was obtained with fraction *A* than with fraction *B* and that a still greater activity was obtained when both enzymes were present indicates that under these circumstances the peroxide-generating enzyme was rate-limiting. These experiments were repeated with the methional-cleaving enzyme isolated from the CM-Sephadex C-50 column and the oxidase enzyme separated after treatment with uranyl acetate. No ethylene was produced by either fraction when glucose (1%, w/v), EDTA (2 mM), cofactor (extract *Z*) and methional (1 mM) in 0·1 M-sodium phosphate buffer were added. However, when both fractions were present 2·2 μl. of ethylene/hr. was readily produced. These results thus confirmed our earlier conclusions that two distinct enzymes are involved in the formation of ethylene from methional.

The minimum concentration of glucose oxidase that had to be added to the methional-cleaving enzyme to generate sufficient peroxide to initiate ethylene production was determined with fungal glucose oxidase. Since the methional-cleaving enzyme preparation used in this experiment was not completely free from the plant oxidase, it was necessary to determine the additional ethylene produced as a result of adding known amounts of glucose oxidase. The relationship between the glucose oxidase activity added (and therefore the additional peroxide-forming capacity) and the additional ethylene formation is shown in Fig. 2. There was a linear relationship between peroxide-generating capacity and ethylene production when concentrations of oxidase capable of generating only very small amounts of peroxide were used. Under such conditions 1 μmole of ethylene was formed when 3 μmoles of peroxide were produced, corresponding to the utilization of three atoms of oxygen/mol. of ethylene formed (Fig. 2). With higher concentrations of oxidase, the ratio of ethylene produced to peroxide formed was lower, which suggests that peroxide formed in excess of that required for the above reaction decomposed or was utilized in other reactions. The corresponding values for peroxide produced and ethylene formed by the enzymes in the natural extract are given in

Table 2 (Expt. 1). These values were obtained from enzyme concentrates prepared by precipitation with ammonium sulphate, because it was impossible to determine the oxidase activity with any accuracy in the non-particulate fraction. The ratio of peroxide produced to ethylene formed was somewhat higher than that shown above, and this we attribute to utilization of peroxide in other reactions, e.g. oxidation of phenols (see below) present in the extracts used to supply cofactor.

These results are in agreement with our previous finding (Mapson & Wardale, 1967) that peroxide formation was essential for the activity of the methional-cleaving enzyme. The present results strongly suggest that the enzyme present in the extracts of the florets was a glucose oxidase, though they do not prove it conclusively; such proof must await the isolation and characterization of the pure protein. That the peroxide formed during the oxidation of glucose was the effective agent was also indicated by the fact that the other known products of the reaction, D-gluconic acid or its lactone, were incapable themselves of forming ethylene in the presence of the methional-cleaving enzyme and its cofactor.

Oxygen partial pressure. The relation between oxygen partial pressure and ethylene production by this enzyme system is also consistent with the participation of a glucose oxidase. Extracts containing both the methional-cleaving enzyme and the natural oxidase were examined for their ability to convert methional into ethylene under different oxygen partial pressures. The results (Table 2) show that ethylene production and oxygen uptake in the glucose oxidase test were similarly affected by altering the atmosphere from 5 to 100% oxygen. The rate of ethylene formation in air was only 60% of that in 100% oxygen and much less (20%) in an atmosphere of 5% oxygen. The corresponding data indicating the relationship between oxygen partial pressure and the activity of fungal glucose oxidase, taken from the results of Keilin & Hartree (1948), are 44% in air and 11–12% in 5% oxygen. Since the methional-cleaving enzyme will produce ethylene from methional in the complete absence of oxygen provided that hydrogen peroxide is added, the effect of increasing the partial pressure of oxygen on the rate of the reaction must be attributed to its effect on the activity of the plant oxidase.

Ethylene reaction initiated by fatty acid hydroperoxide

The utilization of hydrogen peroxide by the methional-cleaving enzyme to catalyse the breakdown of methional to ethylene prompted us to determine the specificity of the enzyme towards another peroxide of physiological interest, the hydroperoxide of linolenic acid. For this purpose we replaced the glucose–glucose oxidase by a linolenic acid–lipoxidase enzymic system and used the latter in conjunction with methional, a methional-cleaving enzyme and cofactors. The results (Table 3) show clearly that with this system ethylene was produced but that the omission of any one of fatty acid, methional, methional-cleaving enzyme or cofactor prevented the reaction. When lipoxidase was omitted, ethylene was formed if the methyl ester of linolenic acid had previously been 'aged' by exposure to oxygen with subsequent formation of hydroperoxide (recognized by absorption at 234mμ). When non-conjugated linolenic acid was substituted, ethylene was only produced in the presence of added lipoxidase. These experiments thus demonstrate that hydroperoxides of linolenic acid, whether produced by autoxidation or under the influence of a lipoxidase enzyme, could replace hydrogen peroxide in the production of

Table 2. *Relationship between oxygen partial pressure and ethylene production*

Flasks contained both enzymes, isolated from sucrose–phosphate extracts by precipitation with 30–60%-saturated $(NH_4)_2SO_4$, in 0·1 M-sodium phosphate buffer, pH 6·8, glucose (1%, w/v), EDTA (2 mM), cofactor (extract *Y* in Expt. 1; extract *Z* in Expt. 2) and methional (1 mM), in a total volume of 10 ml. Flasks were incubated at 25° with shaking after being flushed with the respective gas. Determinations of oxygen uptake were carried out on the same amount of enzyme fraction in 0·1 M-sodium phosphate buffer, pH 6·8, at 25° with the addition of catalase (0·1 ml.) and glucose (1%, w/v), after flushing with the respective gas. —, Not determined.

		Ethylene formed		Oxygen uptake	
		(μl./hr.)	(% of that in oxygen)	(μl./hr.)	(% of that in oxygen)
Expt. 1	Air	3·4	63	8	53
	Oxygen	5·4	(100)	15	(100)
Expt. 2	5% Oxygen	0·3	20	—	—
	Air	0·86	58	—	—
	Oxygen	1·48	(100)	—	—

Table 3. *Production of ethylene by fatty acid hydroperoxides*

Flasks contained the methional-cleaving enzyme, after elution from CM-Sephadex C-50, in 0·1 M-sodium phosphate buffer, pH 6·8, EDTA (2 mM), cofactor (extract *Z*), methional (1 mM), methyl linolenate (10 mM) and lipoxidase (1 mg.), in a total volume of 10 ml. Flasks were incubated at 25° with shaking.

Expt. no.	System	Time (hr.) ...	Ethylene formed (μl.) 0·5	1	2	4·5
1	Complete system with peroxidized methyl linolenate		1·44	2·22	3·05	4·4
	No methional-cleaving enzyme		0	0·07	0·12	0·24
	No methional		0	0	0	0
	No cofactor		0	0	0	0
	No peroxidized methyl linolenate		0	0	0	0
	No lipoxidase		0·62	1·2	2·16	3·0
2	Complete system with non-peroxidized methyl linolenate		—	0·08	0·31	1·0
	No lipoxidase		0	0	0	0

ethylene from methional. Experiments with linoleic acid or arachidonic acid showed that they could initiate the ethylene reaction, though they were much less effective; oleic acid was completely inactive.

We have moreover shown that a protein possessing lipoxidase activity could be prepared from an aqueous extract of floret tissue by precipitation with ammonium sulphate (0–70% saturation, 47·2 g./100 ml.) and dialysis against 0·1 M-sodium phosphate buffer, pH 6·8, to remove diffusible components. Enzyme activity was measured by determining the oxygen uptake of this fraction in the presence of methyl linolenate and comparing this with controls consisting of (1) heat-inactivated enzyme with fatty acid and (2) the enzyme fraction alone. In all cases EDTA (2 mM) was present to eliminate autoxidation due to trace metals. The oxygen uptake of the controls was 2 μl./hr. and 7 μl./hr. respectively, compared with an uptake of 24 μl./hr. with the complete system. Assuming that the extracted enzyme has a similar activity *in vivo*, this corresponds to the formation of hydroperoxide at a rate of 45 μmoles/kg./hr. in the floret tissue.

Inhibition of the enzymic system by dihydroxyphenols

In extracts prepared from the florets, the production of ethylene did not follow a linear course. Initially there was a phase of low activity, which could be as short as 1–2 hr. or could last for as long as 24 hr., and which preceded one of much greater activity. Occasionally we made extracts from some cauliflowers in which this phase of low activity persisted throughout the experiment. As shown by Mapson & Wardale (1967), this phase could always be terminated by the addition of glucose oxidase. Fig. 3 shows the effect of adding increasing amounts of fungal glucose oxidase to extracts containing methional, the methional-cleaving enzyme and

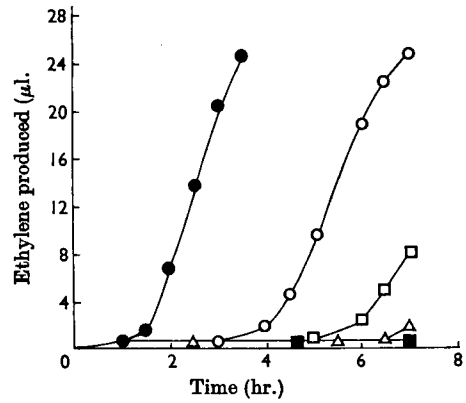

Fig. 3. Effect of glucose oxidase in shortening the lag phase. The methional-cleaving enzyme fraction was precipitated with 30–60%-saturated $(NH_4)_2SO_4$ and dialysed for 16 hr. against 0·1 M-sodium phosphate buffer, pH 6·8. Flasks contained the enzyme fraction, EDTA (2 mM), glucose (1%, w/v), cofactor (extract *X*) and methional (1 mM) in 0·1 M-sodium phosphate buffer, pH 6·8, in a total volume of 10 ml. Fungal glucose oxidase was added as follows: ●, 5 μg.; ○, 2·5 μg.; □, 1·5 μg.; △, 1 μg.; ■, nil.

cofactor in shortening the length of the lag phase. As the peroxide-generating activity was increased so was the length of the phase of low activity diminished.

We suggested previously (Mapson & Wardale, 1967) that these observations might result from the absence from the freshly prepared extract of a peroxide-generating system, which might develop as the extract aged. This has been shown to be incorrect. Assays of glucose oxidase activity in extracts that show this phenomenon indicated that the enzyme was present in as great a concentration

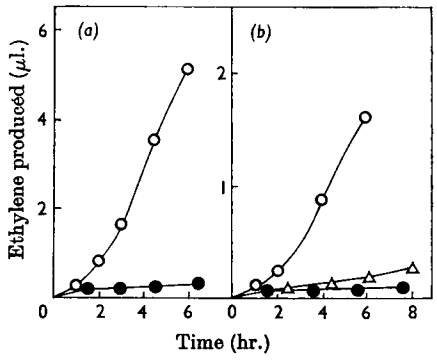

Fig. 4. Removal of inhibitors in the cofactor extracts by (*a*) oxidation and by (*b*) adsorption on polyamide. The fraction containing both the methional-cleaving enzyme and the peroxide-generating enzyme was precipitated by 30–60%-saturated $(NH_4)_2SO_4$ and dialysed for 16hr. against 0·1M-sodium phosphate buffer, pH6·8. Flasks contained the enzyme fraction, EDTA (2mM), glucose (1%, w/v) and methional (1mM) in 0·1M-sodium phosphate buffer, pH6·8, in a total volume of 10ml., with the following additions. (*a*) ●, Untreated cofactor (extract *X*); ○, cofactor extract bubbled with O_2 for 16hr. at 25° before heating (extract *Y*). (*b*) ○, Components in boiled aqueous extract not adsorbed on polyamide at pH8·0; ●, components adsorbed on polyamide and eluted with 2N-NH_3; △, both components.

during the lag phase as in the subsequent phase of greater activity. It appeared from such results that the enzyme generating peroxide was present and was apparently fully functional. The explanation of why the reaction exhibits this latent phase must therefore reside elsewhere.

One possibility was that though peroxide was being produced during this early phase it was preferentially reacting with other substances present in the extracts. This we have now found to be the case, and we have provisionally identified phenolic compounds as those most likely to be involved. Substances inhibiting the reaction were shown to be present in the heated aqueous extract from the florets, which were added to provide the cofactor for the methional-cleaving enzyme. That these inhibitory substances were removed by oxidation is shown by the fact that exposure to oxygen decreased the duration of the phase of low activity (Fig. 4). Moreover, most of these inhibitory compounds could be removed if the extract (pH8·0) was passed through a column of polyamide or Polyclar, compounds that adsorb phenols. This operation did not appreciably diminish the concentration of the cofactor but did largely remove the inhibitory compounds, as shown by the shortening of the lag phase in the experiment illustrated in Fig. 4(*b*). Elution of the inhibitory substances could

Table 4. *Inhibitory effect of monohydroxy- and dihydroxy-phenols*

Flasks contained the enzyme fraction, precipitated by 30–60%-saturated $(NH_4)_2SO_4$ and dialysed for 16hr. against 0·1M-sodium phosphate buffer, pH6·8, EDTA (2mM), cofactor (extract *Z*), glucose (1%, w/v), fungal glucose oxidase (10μg.), methional (1mM) and the phenol to be tested, in 10ml. of 0·1M-sodium phosphate buffer, pH6·8.

Phenol	Concn. (mM)	Extension of lag phase (hr.)
Cinnamic acid	1·0	0
Ferulic acid	1·0	24
Caffeic acid	1·0	>24
p-Coumaric acid	1·0	1
Gallic acid	1·0	4
Phenol	1·0	0
Resorcinol	1·0	> 7
Catechol	1·0	>24
Pyrogallol	1·0	3
Phloroglucinol	0·1	0
Chlorogenic acid*	0·1	> 5
Sinapic acid*	0·1	> 5

* No fungal glucose oxidase added.

be achieved with 2N-ammonia. If the aqueous extract was reconstituted by the addition of the ammonia eluate to the non-adsorbed fraction, restoration of the lag phase was observed (Fig. 4*b*). Similar experiments showed that the inhibitory compounds separated on Sephadex were of low molecular weight; this, combined with the observation that if the compounds were oxidized by exposure to pure oxygen at pH9·0 their inhibitory properties were lost, pointed to the same conclusion. We have been able to show that the addition of certain dihydroxyphenols prolonged the duration of the phase of low activity. Further, the inhibitory effect of such phenols was reversed or partially reversed by the addition of exogenous glucose oxidase. The ability of the enzyme extracts to oxidize phenols also ran parallel with the ability of the latter to inhibit the formation of ethylene from methional. Thus in the experiments (Table 4) in which the inhibitory effect of certain simple phenols was tested by determining the time-interval elapsing before the rapid onset of ethylene formation, the marked inhibition observed with both ferulic acid and caffeic acid was in sharp contrast with the failure of mono-hydroxylated cinnamic acid to inhibit. Similarly the inhibition observed with the dihydroxyphenols catechol and resorcinol was in contrast with the lack of inhibition by phenol.

Separate experiments also established that the phenols chlorogenic acid and sinapic acid were inhibitory even at concentrations of 0·1mM; the latter phenol has been identified in the extract

made from the florets. Both these phenols were rapidly oxidized in the presence of enzyme extract and hydrogen peroxide, but neither was oxidized in the absence of either enzyme or peroxide. These latter observations indicate (1) that these dihydroxyphenols are enzymically oxidized in the presence of hydrogen peroxide and (2) that they inhibit the formation of ethylene from methional by competing with the methional-cleaving enzyme for the peroxide generated by the plant oxidase.

These dihydroxyphenols likewise inhibited the production of ethylene when the lipoxidase–linolenate system replaced that of the glucose oxidase–glucose system.

Some properties of the methional-cleaving enzyme

Flavine. Abeles & Rubinstein (1964) first reported that FMN produced ethylene from an unknown substrate in etiolated pea hypocotyls. Yang, Ku & Pratt (1966) identified the substrate as methionine and noted that, though light was necessary to catalyse the reaction mediated by the flavine, an enzyme was not involved. Mapson & Wardale (1967) found that flavines (FMN and FAD) catalysed the formation of ethylene from methional, but they excluded free flavines from being responsible for the ethylene production from floret tissue because (1) no free flavines could be detected in their active extracts and (2) the enzymic reaction was insensitive to EDTA, whereas that catalysed by the flavines was inhibited. Nevertheless the possibility existed that flavine might be a constituent of the prosthetic group of the methional-cleaving enzyme. An attempt was therefore made to determine if the enzyme contains flavine. The enzyme was subjected to conditions under which flavine is dissociated from many flavoproteins, i.e. the enzyme was precipitated after acidification to pH 1·8 from a saturated ammonium sulphate solution. The precipitate, separated by centrifugation, was dissolved in 0·1 M-sodium phosphate buffer, pH 6·8, and dialysed for 4 hr. against the same buffer. This treatment did not impair the activity of the enzyme, neither was its activity increased by the addition of either FMN or FAD, after removal of excess of these components by dialysis. Thus no evidence was obtained that the enzyme contained flavine.

Substrate specificity. Methional is, as yet, the only substrate found from which the enzyme will produce ethylene. Methionine, ethionine and *S*-methylcysteine were not converted, and the same applies to acrolein, and 3-methylthiopropionic acid and its esters. The failure of these latter compounds to act as substrates showed that they could not be considered as intermediates in the breakdown of methional to ethylene. The inability of 3-methylthiopropionic acid to act as substrate was in accordance with our observations of the production of ethylene from methional in model systems (Lieberman, Kunishi, Mapson & Wardale, 1965). The corresponding alcohol (3-methylthiopropanol) was also inert, from which results it is clear that the aldehyde group is essential. The related aldehydes acetaldehyde, propionaldehyde and butyraldehyde could not, however, serve as substrates, nor did they act as competitive inhibitors.

Table 5. *Effect of chelating agents*

The methional-cleaving enzyme was eluted from CM-Sephadex C-50. All flasks contained the enzyme fraction in 0·1 M-sodium phosphate buffer, pH 6·8, EDTA (2 mM), cofactor (extract *Z*), hydrogen peroxide and methional (1 mM), in a total volume of 10 ml. Flasks were incubated at 25° with shaking. The chelating agents, DIECA (sodium diethyldithiocarbamate), cuprizone (biscyclohexanone oxalyldihydrazone), bathocuproine disulphonic acid (2,9-dimethyl-1,4-diphenylphenanthroline-1,10-disulphonic acid), homocysteine, cysteine or azide, were left with the enzyme for 5 min. before the addition of the other reagents.

Chelating agent	Hydrogen peroxide added (μmoles/ml.)	Reaction time (min.)	Inhibition (%) 15	30	45
DIECA (1 mM)	2·0		23	0	0
DIECA* (1 mM)	2·0		47	32	26
DIECA (1 mM)	0·5		40	40	40
DIECA (1 mM)	0·2		100	100	100
Cuprizone (0·1 mM)	2·0		79	81	81
Bathocuproine (0·1 mM)	2·0		67	66	66
Homocysteine (1 mM)	2·0		96	94	83
Cysteine (1 mM)	2·0		77	46	46
Azide (1 mM)	2·0		55	61	63

* The enzyme fraction and DIECA in 0·1 M-sodium phosphate buffer, pH 6·8, were dialysed against the same buffer for 5 hr. before the addition of the other reagents. A control sample without inhibitor was treated in a similar manner.

Inhibitors. Previously (Mapson & Wardale, 1967) we described experiments in which the activity of enzyme extracts producing ethylene from methional was severely inhibited by reagents chelating with copper. We have re-examined the activity of these compounds on the purified methional-cleaving enzyme. To eliminate the influence of these reagents on the plant oxidase the reaction was studied by adding hydrogen peroxide in place of that generated by the plant or fungal oxidase. The results (Table 5) show that the two copper reagents cuprizone (biscyclohexanone oxalyldihydrazone) and bathocuproine disulphonic acid (2,9-dimethyl-4,7-diphenylphenanthroline-1,10-disulphonic acid) inhibited strongly at a concentration of 0·1 mM with peroxide added at an initial concentration of 2 μmoles/ml. Sodium diethyldithiocarbamate (1 mM) under similar conditions also inhibited but to a smaller degree. However, it was noted that with this concentration of peroxide (2 μmoles/ml.) the inhibition by the dithiocarbamate decreased as the reaction proceeded. With lower concentrations of peroxide (0·5–0·2 μmole/ml.) the initial degree of inhibition was higher, and it remained constant throughout the experiment (Table 5). The decrease in the inhibition with the higher concentration of peroxide appears to be due to the fact that the dithiocarbamate was decomposed in the presence of hydrogen peroxide, and this in turn raises the question whether the effect of this reagent in inhibiting the methional reaction is due simply to its reaction with peroxide, thus lowering the concentration of the latter so that the methional reaction is slowed down. An attempt was made to test this hypothesis by first allowing the dithiocarbamate to react with the methional-cleaving enzyme, removing excess of the chelating agent by dialysis and then testing for enzyme activity. In these circumstances any inhibition observed must presumably be due to inactivation of enzyme and any reactivation must then be due to decomposition by peroxide of the enzyme–thiocarbamate complex. As the experiment in Table 5 shows, marked inhibition occurred in the first 15 min. of the reaction, but it slowly decreased as the reaction proceeded. It seems fair to interpret these results as being due to the formation of an inactive enzyme–thiocarbamate complex that is decomposed in the presence of higher concentrations of peroxide. Compounds possessing free thiol groups, e.g. homocysteine and cysteine, behaved in a similar manner. Azide, which is not affected by peroxide, inhibited, and this inhibition remained constant throughout the experiment. Generally these results suggest that the prosthetic group of the methional-cleaving enzyme contains copper, though iron cannot be excluded on these grounds alone.

Thiol reagents. Reagents reacting with thiol groups had no effect on the methional-cleaving enzyme. *N*-Ethylmaleimide, *p*-chloromercuribenzoate and iodoacetamide, when used in concentrations from 0·1 mM to 1 mM, had no effect whatsoever on the enzymic activity. It seems therefore that a free thiol group is not involved in the reaction sequence.

DISCUSSION

We have given details in this paper of the separation and partial purification of an enzyme essential for the formation of ethylene from methional. The evidence at present available indicates it is a protein with a molecular weight of about 150000, including copper or iron in its prosthetic group. It does not contain flavine and is not dependent on the integrity of any thiol groups for its activity. The enzyme catalyses the formation of ethylene from methional in the presence of either hydrogen peroxide or of a hydroperoxide formed by oxidation of linolenic acid. Besides the integrity of the aldehyde group of the substrate, methional, the presence of two cofactors is necessary.

We have also shown the presence in cauliflower extracts of a plant oxidase and have separated it from the methional-cleaving enzyme. This oxidase had properties akin to the glucose oxidase of fungal origin. Thus its specificity for its substrate D-glucose and its affinity for oxygen were similar to those reported for the fungal enzyme. Its activity in floret tissue was low, so that it was difficult to measure it accurately in simple extracts unless they were first concentrated. On the basis of assays on concentrates it can be calculated that the oxidase in the fresh floret tissue could generate about 36 μmoles of hydrogen peroxide/kg./hr. at 25°, which is adequate to sustain a known rate of ethylene production of 0·1 μmole/kg./hr. at the same temperature. In addition, we have obtained evidence of the presence of a lipoxidase that in the presence of linolenic acid or other unsaturated acids could generate hydroperoxides capable of initiating ethylene formation. The rate of production of hydroperoxide with the extracted lipoxidase enzyme from floret tissue has been calculated to be of the same order of magnitude as the rate of formation of hydrogen peroxide by the plant oxidase. One might therefore expect the generation of ethylene to be comparable when the reaction was initiated with either peroxide, unless the affinity of the methional-cleaving enzyme for the two peroxides was different. In one experiment, in which a direct comparison was made between the efficiency of glucose or methyl linolenate as substrates for initiating the production of ethylene from methional in the presence of the natural

cofactors and enzymes, the phase of low activity was shorter and the rate of subsequent production of ethylene much greater with glucose than with the fatty acid. Though these results obtained *in vitro* suggest that hydrogen peroxide is more efficient than hydroperoxide in the enzyme reaction with methional in floret tissue, other factors operating *in vivo* may modify these activities. Which, if either, of these enzymes forming peroxide is the most important physiologically therefore remains to be determined. The possibility of the participation of a lipoxidase enzyme in the biogenesis of ethylene is of interest in view of the observations of Wooltorton, Jones & Hulme (1965) that the increase in the concentration of this enzyme in the peel tissue of the apple coincides with the phase of rapid ethylene production that is associated with the climacteric in the fruit.

Note added in proof. While this paper was in the press a report by Yang (1967) appeared describing a model system in which horseradish peroxidase in the presence of hydrogen peroxide and other cofactors (sulphite and phenols) produced ethylene from methional. We have found that the methional-cleaving enzyme, isolated as described above, does indeed possess peroxidative activity as tested by its catalytic effect in the peroxidase–purpurogallin test of Sumner & Gjessing (1943). It is possible therefore that the methional-cleaving enzyme here isolated may in fact be a peroxidase. In support of this it is possible to substitute horseradish peroxidase for the methional-cleaving enzyme and produce ethylene from methional. Further, the peroxidative activities of the enzyme from the florets, or of horseradish peroxidase, correlated exactly with their ability to catalyse the production of ethylene from methional. On the basis of the experiments with the copper reagents, we suggested that our enzyme contained copper. It may still be so, but alternatively further examination may show it to be an orthodox iron–proteinate peroxidase.

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