Biosynthesis of Ethylene

METHANESULPHINIC ACID AS COFACTOR IN THE ENZYMIC FORMATION OF ETHYLENE FROM METHIONAL

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The second cofactor in the peroxidase-catalysed formation of ethylene from methional in cauliflower extracts was identified as methanesulphinic acid. The progress of the reaction is described and the activities of related sulphinic acids were determined.

The production of ethylene from methional in extracts from cauliflower florets is due to the operation of two enzymes, a glucose oxidase and a peroxidase (Mapson & Wardale, 1968). The hydrogen peroxide produced by the first enzyme is utilized by the second in the presence of two cofactors to cleave methional with the formation of ethylene. The reactions may be formulated as follows:

The first of these cofactors has been identified as an ester of *p*-coumaric acid (Mapson & Mead, 1968) and the second has now been isolated as its trimethylsilyl derivative and identified as methanesulphinic acid.

EXPERIMENTAL.

Isolation of methanesulphinic acid. The acid was isolated as the trimethylsilyl derivative after fractionation from aqueous extracts of floret tissue. The preliminary stages in the purification were modified from the procedure published by Mapson & Mead (1968). After removal of cations by passage through Zeo-Karb 225 resin (H^+ form) the extract was reneutralized to pH 6–7.0 with aq. NH_3 . This procedure was necessary because of the extensive decomposition of the acid that occurred when acid extracts were concentrated *in vacuo*; the ammonium salt is stable and little loss occurs during this stage with the acid in this form. The residue was acidified with 2.5N-HCl in 80% (v/v) ethanol to adjust the pH to approx. 2.0 and the solution immediately extracted three times with 3 vol. of chloroform. The free acid is stable in this solution and was recovered as the ammonium salt by extraction of the chloroform solution with 2N- NH_3 . After removal of excess of NH_3 many of the

phenolic compounds were removed by treatment with polyamide as described by Mapson & Mead (1968).

Further purification was achieved either (1) by paper chromatography with propan-1-ol-aq. NH_3 (sp.gr. 0.88) (7:3, v/v) as described by Mapson & Mead (1968) or (2) by gradient elution with formic acid from Dowex 1 (X8) resin, followed by chromatography on silica-gel columns by the method of Wager & Isherwood (1961). Final purification was achieved by preparative gas-liquid chromatography on silica gel, details of which were also given by Mapson & Mead (1968).

Sources of sulphinic acids. Methanesulphinic acid was prepared from methanesulphonyl chloride by the method described by Wudl, Lightner & Cram (1967), the sulphonyl chloride being itself prepared from dimethyl disulphide by the procedure of Douglass, Farah & Thomas (1961). The acid was recrystallized (m.p. 41.5°) from ether.

Benzenesulphinic acid (sodium salt) was purchased from British Drug Houses Ltd. (Poole, Dorset). Cysteine-sulphinic acid was purchased from Sigma (London) Chemical Co. (London, S.W. 6).

Combined gas-liquid chromatography and mass spectrometry. The mass spectrum of the trimethylsilyl derivative was examined by combined gas-liquid chromatography and mass spectrometry. The apparatus and method of operation have been described by Self (1967). A sample (0.5 μl .) of the total reaction mixture containing the trimethylsilyl derivative and prepared as described by Mapson & Mead (1968) was injected into a 50 ft. length of 0.02 in. (internal diam.) support-coated open tubular column containing SE30 (manufactured by Perkin-Elmer Ltd., Beaconsfield, Bucks.) as stationary phase. The column was temperature-programmed from 80° at 6°/min. The unknown trimethylsilyl compound was eluted on the tailing edge of the pyridine solvent peak. Earlier experiments on the same phase provided the retention data required to establish which one of the three peaks eluted after the solvent contained the active compound. Three fast-scan spectra were taken during the elution period to provide sufficient data to enable the 'unknown' spectrum to be subtracted from the interfering 'background' spectrum produced by the solvent and the other two small components. The mass spectra were obtained on an Edwards 60° mass spectrometer with a 70 eV electron beam, a 1 sec./decade voltage scan from 5 kv to

500 v (25–250 atomic mass units) and an inlet temperature of 120°. The spectra were taken at maximum intensity as shown by an oscilloscope monitor, and no significant change in the relative intensity of the ions occurred in the three spectra obtained.

Enzymic tests. The enzymic tests used to determine the activity of the unknown and other acids were carried out as described by Mapson & Wardale (1968), except that horseradish peroxidase was used in place of the peroxidase from the floret tissue. The peroxidase enzyme (0.3 purpurogallin unit) was added to 10 ml. of the reaction solution.

RESULTS

The observation that the unknown acid prepared from its trimethylsilyl derivative contained sulphur was indicated by positive reactions in the tests for the element described by Feigl (1966). Moreover the indication from such tests that the compound contained either bi- or quadri-valent sulphur, and that the acid on treatment with bromine was converted into a compound containing 'active' halogen (since after bromine treatment it immediately liberated iodine from hydroiodic acid), suggested the possibility of a sulphinic acid derivative. The destruction of its enzyme-promoting activity after treatment with bromine or other oxidizing agents, e.g. permanganate, was also consistent with this view.

Gas-liquid chromatography. A comparison between the temperature of emergence of the trimethylsilyl derivatives of acetic acid and the unknown acid (108° and 134°) on the one hand, and of benzoic acid and benzenesulphinic acid (188° and 211°) on the other, showed a similar increase in elution temperature when the sulphinic group replaced the carboxyl group. This suggested that the unknown acid was methanesulphinic acid. The trimethylsilyl derivatives of the unknown acid and of authentic methanesulphinic acid were prepared and examined by gas-liquid chromatography on an SE 30 column temperature-programmed from 100° at 4°/min. The temperature of emergence (134°) was the same for both acids, they formed a single peak on the chromatogram when co-chromatographed and the peak areas in relation to enzymic activity were also identical for both acids.

Paper chromatography. The ammonium salts of the unknown acid and methanesulphinic acid were chromatographed and co-chromatographed with propan-1-ol-aq. ammonia (sp.gr. 0.88) (7:3, v/v); both had the same R_F value (R_F 0.5). With butan-1-ol-aq. ammonia (sp.gr. 0.88) (4:1, v/v) both compounds again had the same R_F value (R_F 0.10). Attempts to chromatograph the acids in acid solvents were unsuccessful, no doubt because of the instability of the free acids.

Mass spectrometry. The volatile trimethylsilyl derivative was chosen for this work because the

Table 1. *Mass spectrum of the trimethylsilyl ester of methanesulphinic acid*

The mass spectrum was obtained from the Edwards 60° mass spectrometer: inlet temperature 120°, electron energy 70 ev, 1 sec. voltage scan (m/e 25–300).

m/e	Relative intensity (%)	m/e	Relative intensity (%)
27	1.0	60	1.8
28	2.1	61	5.0
29	3.7	63	3.4
31	1.6	72	1.4
42	1.0	73	100.0
43	8.0	74	8.8
44	2.8	75	38.4
45	20.0	76	3.0
46	2.5	77	3.2
47	6.6	93	2.4
48	1.2	95	1.5
55	1.6	122	3.0
57	1.0	137	24.8
58	6.0	138	3.0
59	8.0	139	1.8
		152	7.3

main task was to determine an accurate molecular weight. Although these derivatives do not normally have a significant molecular ion in their mass spectrum they normally produce an intense $M - 15$ ion due to methyl loss from the tertiary silicon centre (Sharkey, Friedel & Langer, 1957). The mass spectrum shown in Table 1 has a strong ion at m/e 137 (24.8%) and this was taken to be the $M - 15$ species. The ion at m/e 152 (7.3%) was assumed to be the molecular ion, giving the unknown acid a molecular weight of 80. The ion at m/e 122 (3%) was assumed to be due to further loss of methyl, which was tentatively taken to indicate the presence of a methyl group in the unknown acid. No further structural information could be derived from the mass spectrum. Among the remaining ions were the two characteristic series of ions produced from trimethylsilyl compounds, namely 31, 45, 59 and 73 and 47, 61 and 75 (Beynon, Saunders & Williams, 1968).

On the assumption that the trimethylsilyl derivative is formed by reaction with a hydroxyl group and taking into account the presence of sulphur, the molecule was considered to contain a methyl, a tetravalent sulphur and a hydroxyl group, leaving one atom of oxygen to give the molecular weight determined by mass spectrometry. The atomic composition therefore appeared to be $\text{CH}_4\text{O}_2\text{S}$. A knowledge of the chemical characteristics of the unknown acid suggested a molecular configuration of $\text{CH}_3\cdot\text{S}(\text{O})\cdot\text{OH}$, methanesulphinic acid. In addition, subsequent tests on authentic

Table 2. Activity of related sulphinic acids and sulphite

The concentration of *p*-hydroxybenzoic acid was 0.075 mM in all experiments, and that of glucose oxidase was 1 μ g./ml. in Expt. 1 and 0.5 μ g./ml. in Expt. 2.

	Sulphur-containing acid	Concn. (mM)	Rate of ethylene production (μ l./30 min.)
Expt. 1	Methanesulphinic acid	0.075	5
	Benzenesulphinic acid	0.075	2.15
	NaHSO ₃	0.075	1.9
Expt. 2	Methanesulphinic acid	0.5	16.0
	Benzenesulphinic acid	0.5	5.9
	Cysteinesulphinic acid	0.5	0.7
	NaHSO ₃	0.5	5.4

methanesulphinic acid showed a mass spectrum identical with that of the unknown acid.

Like the free acid the trimethylsilyl derivative appears to be unstable once separated from excess of the silyl reagents. Attempts made to trap and rechromatograph the compound after separation from solvent and reagents were unsuccessful. This instability may account for the intense ion at *m/e* 73 in the mass spectrum, attributed to loss of the trimethylsilyl group.

Wudl *et al.* (1967) obtained the mass spectrum of the free acid by using a direct-inlet system at room temperature. The base peak of this spectrum occurred at *m/e* 65 (*M* - 15), and they observed that at a higher inlet temperature of 50° the molecular ion at *m/e* 80 was decreased from 50% relative intensity to almost zero.

Activity of sulphinic acids and sulphite ion. The effect of replacing the methyl group of the sulphinic acid by other groups, e.g. aryl and cysteinyl, was examined, and their activities were compared with that of the sulphite ion. These tests were carried out by using the standard enzyme reaction mixture (volume 10 ml.) containing 0.75–5 μ moles of each of the acids with *p*-hydroxybenzoic acid (3.0 μ moles) as the phenolic cofactor. The most efficient of the three sulphinic acids tested was the methyl derivative, the rate of ethylene production being about twice that obtained with benzenesulphinic acid and 25 times that with cysteinesulphinic acid (Table 2). The rate of the reaction catalysed by the sulphite ion was about one-third of that observed with methanesulphinic acid.

There was also a difference between sulphite and sulphinic acids in their ability to promote the reaction in the presence of different phenols. Thus neither methanesulphinic acid nor benzenesulphinic acid yielded ethylene when either resorcinol or phenol replaced *p*-hydroxybenzoic acid, whereas

Fig. 1. Formation of ethylene from methional with *p*-hydroxybenzoic acid and methanesulphinic acid as cofactors. The solution contained glucose oxidase (5 μ g.), glucose (1%, w/v), horseradish peroxidase (0.3 purpurogallin unit) with (where indicated) *p*-hydroxybenzoic acid (7 μ moles) and methanesulphinic acid (6 μ moles), with methional (1 mM), in a volume of 10 ml. of 0.1 M-sodium phosphate buffer, pH 6.5, containing EDTA (1 mM). After 3 hr. *p*-hydroxybenzoic acid or methanesulphinic acid or both were added in the same amounts as used initially. □, *p*-Hydroxybenzoic acid and methanesulphinic acid; ○, methanesulphinic acid; Δ, *p*-hydroxybenzoic acid.

with sulphite the rate of reaction was essentially the same with all three phenols. Similarly with chlorpromazine, which forms a stable free radical at pH 4.5 (Piette, Bulow & Yamazaki, 1964), methanesulphinic acid reacted readily and benzenesulphinic acid less readily, but sulphite was ineffective.

Progress of the reaction. The progress of the reaction with methanesulphinic acid is illustrated in Fig. 1. Under our experimental conditions the reaction stopped in about 1.5 hr. This appears to be chiefly due to the loss of the sulphinic acid, because the reaction may be reinitiated by the subsequent addition of this acid alone. However, the rate achieved on adding the acid was less than that originally observed unless the phenolic cofactor and

Fig. 2. Interrelation between rates of formation of H₂O₂, formation of ethylene and concentration of *p*-hydroxybenzoic acid. The standard reaction mixture (10ml.) contained various amounts of glucose oxidase and *p*-hydroxybenzoic acid, with a constant amount (6 μmoles) of methanesulphonic acid. Concentration of *p*-hydroxybenzoic acid (μmoles/10ml. reaction volume): ●, 3.5; □, 7.0; △, 14; ○, 35.

peroxidase were also added. There is thus some evidence that during the progress of the reaction there is a loss of the phenolic compound and of peroxidase. There was no evidence that loss of methional or of glucose oxidase activity were in any way responsible for the cessation of the reaction.

The view that the reaction is terminated by the loss of sulphinic acid is supported by the results of the following experiments. Prior incubation of the enzymic system, glucose, glucose oxidase and peroxidase with both the phenolic cofactor and the sulphinic acid for 1 hr. in the absence of methional leads to their oxidation and the complete failure of the system to produce ethylene when methional is added. Prior incubation of *p*-hydroxybenzoic acid alone leads to a marked decline in the rate of ethylene formation on the subsequent addition of the sulphinic acid and methional, but very little

Fig. 3. Interrelation between rates of formation of H₂O₂, formation of ethylene and concentration of methanesulphonic acid. The reaction mixture (10ml.) contained various amounts of glucose oxidase and methanesulphonic acid, with a constant amount (7 μmoles) of *p*-hydroxybenzoic acid. Concentration of methanesulphonic acid (μmoles/10ml. reaction volume): ●, 1.2; □, 3; △, 6; ○, 12.

decrease in rate occurs if the sulphinic acid alone is incubated with the enzyme before the addition of the phenolic cofactor and methional.

The loss of the cofactors is largely determined by the concentration of glucose oxidase and therefore by the rate of production of hydrogen peroxide. With high rates of formation of peroxide both the initial rate and total amount of ethylene produced are less than with lower rates of formation. There is in fact an optimum rate of formation of peroxide that gives the best yield of ethylene. This optimum rate in turn depends on the concentration of the cofactors, principally the phenolic compound. Results showing the relation between rates of reaction, concentration of glucose oxidase and concentration of *p*-hydroxybenzoic acid are shown in Fig. 2. The decline in ethylene production with the lower concentrations of *p*-hydroxybenzoic acid as the rate of peroxide formation is increased is no doubt due to the fact that any excess of peroxide produced over and above that which can be utilized in the ethylene reaction can effect the complete oxidation and consequent inactivation of the phenolic cofactor. Comparable results in which the initial concentration of the phenolic cofactor was constant, but the concentration of the sulphinic acid was varied, are given in Fig. 3. In this case

higher rates of peroxide formation resulted in higher rates of production of ethylene.

These observations, together with the fact that in the complete system the loss of sulphinic acid appears to be the chief cause of the cessation of the reaction, indicate that the oxidation of the sulphinic acid is dependent on the presence of the phenolic cofactor and suggest that the oxidation of the sulphinic acid is coupled with a reduction of the oxidized phenolic cofactor. Thus, while excess of sulphinic acid remains, complete and irreversible oxidation of the phenolic cofactor is prevented.

When peroxide formation is not limiting, the initial rates of reaction depend on both the concentration of the phenolic cofactor and the sulphinic acid, and within the lower concentration range are proportional to the concentration of these compounds. At higher concentrations of the sulphinic acid the reaction was inhibited to a very slight extent. With very low rates of peroxide formation the rate of the ethylene reaction was independent of the concentration of either cofactor.

DISCUSSION

It seems probable that the enzymic production of ethylene from methional by peroxidase involves the formation of free radicals. Yamazaki & Piette (1961), using electron-paramagnetic-resonance techniques, demonstrated the presence of free radicals as intermediates in the oxidation of a number of compounds by peroxidase. Yang (1967) suggested that free-radical formation accounted for the production of ethylene in the model system comprising methional, peroxidase, sulphite and hydrogen peroxide. He and others, including Fridovich & Handler (1961) and Yip & Haley (1966), have shown that the oxidation of sulphite by peroxidase in the presence of hydrogen peroxide was greatly stimulated by the addition of certain phenols. Under these conditions methional inhibited the oxidation of sulphite, and those substances inhibiting ethylene formation from methional also inhibited the oxidation of sulphite. On these grounds Yang (1967) proposed that the phenol is first oxidized by the peroxidase to a phenoxy free radical, which then oxidizes the sulphite ion, the latter reacting with oxygen to produce free radicals of the type $\cdot\text{OH}$. It is these radicals that react with methional to produce ethylene. Our results support the conclusion that the first attack of peroxidase is on the phenolic component, since we have shown that in the absence of the latter the sulphinic acid is not oxidized and no ethylene is produced. Our results further indicate that the reaction is terminated on the loss of the sulphinic acid.

Whether a free radical is involved in the peroxidase-phenolic cofactor-sulphinic acid system

described here remains to be proved. In preliminary experiments in which chlorpromazine, a substance forming a stable red free radical absorbing at $530\text{m}\mu$, replaced the phenolic cofactor, the formation of this free-radical form was observed during the enzymic production of ethylene from methional. Its formation was especially evident at pH 4.5, at which pH it was also most effective in replacing the phenolic cofactor in the system. However, its formation was not observed in the presence of EDTA, despite the fact that ethylene was readily formed.

The question still remains whether the enzymic system described here is the physiological process *in vivo*. It is well established (see Lieberman, Kunishi, Mapson & Wardale, 1966; Burg & Clagett, 1967) that methionine stimulates ethylene production in a number of fruit and vegetable tissues, and tracer experiments have also shown that ethylene is produced from methionine. Methionine is present in cauliflower tissue in quite high concentrations (0.13 g./g. of nitrogen), as shown by McCance & Widdowson (1960), and as Yang (1967) has pointed out methional could easily be formed from methionine by transaminase and decarboxylase enzymes. All the other components of the system, glucose oxidase, peroxidase and the two cofactors, *p*-coumaric esters and sulphinic acid, can be isolated from extracts (Mapson & Wardale, 1968; Mapson & Mead, 1968). Although the concentration of the sulphinic acid is low in fresh extracts it increases after autolysis. This behaviour parallels the low productions of ethylene observed from fresh floret tissue, which increases only after some hours' aging of the tissue (Mapson & Wardale, 1967). All these observations taken together suggest that the enzymic system we have isolated is the system operating *in vivo*. Doubt, however, remains because of our failure, so far, to stimulate ethylene production in the intact florets by the infiltration of methional or the two cofactors or both, or by the addition of either glucose oxidase or peroxidase. Admittedly negative results from such experiments are not conclusive, but failure to achieve positive results must inevitably raise doubts as to the validity of the results in relation to the physiological process.

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