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3-METHYLTHIOPROPIONALDEHYDE PEROXIDASE FROM APPLES: AN ETHYLENE-FORMING ENZYME

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SUMMARY

1. 3-Methylthiopropionaldehyde peroxidase, a 3-methylthiopropionaldehyde-cleavage ethylene-forming enzyme, was isolated from apples, after separation from phenols and polyphenols by dialysis and gel-filtration on Sephadex G-25. The specific activity of 3-methylthiopropionaldehyde peroxidase was increased about 300 times by purification with DEAE- and CM-cellulose.

2. This enzyme was separated into three fractions by free-flow electrophoresis. Each fraction had a different specific activity for the 3-methylthiopropionaldehyde-cleavage and the *o*-dianisidine peroxidase reaction, respectively.

3. The purified enzyme showed a spectral absorption peak in the ultraviolet at 278 nm and a broad peak in the visible spectrum at 410 nm with a shoulder at 525 nm.

4. The enzyme produced ethylene from 3-methylthiopropionaldehyde in a system which required *p*-coumaric acid, HSO_3^- and H_2O_2. This enzyme was inhibited by sodium diethyldithiocarbamate, cupferron, azide, iodoacetamide, *N*-ethylmaleimide, ascorbate, Cu^{2+}, diphenols and catalase. Streptomycin approximately doubled the activity of the 3-methylthiopropionaldehyde-cleavage system.

5. 3-Methylthiopropionaldehyde peroxidase also produced ethylene, in lower amounts, from L-methionine in a two-step reaction. The first step involves a reaction with Mn^{2+}, *p*-coumaric acid, and codecarboxylase. The second step appears to be the 3-methylthiopropionaldehyde-cleavage reaction requiring HSO_3^- and H_2O_2.

INTRODUCTION

Ethylene production from methionine and 3-methylthiopropionaldehyde has been shown[1,2] in model systems. These reactions require either H_2O_2 or free radicals generated by an FMN–light system as intermediate reactants. Recently MAPSON AND WARDALE[3] reported the presence of an enzyme which can produce ethylene from 3-methylthiopropionaldehyde in extracts prepared from cauliflower florets. A peroxide-generating system was required for this reaction. MAPSON AND WARDALE[4] also

purified this enzyme, and it was later shown that the methyl ester of *p*-coumaric acid or *p*-hydroxy benzoic acid were cofactors[5] in this reaction. Two separate enzymes are involved in the 3-methylthiopropionaldehyde-cleaving system; the first generates peroxide under aerobic conditions, and the second uses this peroxide to split 3-methylthiopropionaldehyde. The formation of ethylene from 3-methylthiopropionaldehyde in a model system containing horseradish peroxidase, Mn^{2+} or H_2O_2, HSO_3^-, and resorcinol, has also been reported[6].

Isolation of an ethylene-forming enzyme from apples, a tissue which produces large quantities of ethylene, has not been obtained previously. This is due to high concentrations of phenols and pectin in apple tissues, which interfere in the extraction and subsequent purification procedures. In this report we describe a procedure for isolating 3-methylthiopropionaldehyde peroxidase, a 3-methylthiopropionaldehyde-cleaving (ethylene-forming) enzyme from apples. Some characteristics of this enzyme are described and data are presented which serve to categorize this enzyme as a peroxidase.

MATERIALS AND METHODS

Apples

Rome Beauty apples were purchased from an apple grower and stored at 0° until used.

Enzymes and chemicals

Horseradish peroxidase (400 units/mg), and catalase (3000 units/mg) were purchased from Worthington Biochemical Co., Freehold, N.J., and glucose oxidase (90 000 units/g, Type IV) was purchased from Sigma Chemical Co., St. Louis, Mo. 3-Methylthiopropionaldehyde was prepared from acrolein and methanethiol by the method described by PIERSON, GIELLA AND TISHLER[7]. The DEAE- and CM-cellulose used was DE 23 and CM 23 made by W and R Balston, England. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, N.J.

Assay of ethylene

A standard incubation mixture contained enzyme solution, 10^{-3} M 3-methylthiopropionaldehyde, $2 \cdot 10^{-4}$ M *p*-coumaric acid, $9 \cdot 10^{-4}$ M streptomycin sulfate, $2 \cdot 10^{-4}$ M Na_2SO_3, $7.6 \cdot 10^{-6}$ M H_2O_2, or as substitute for H_2O_2, 1% glucose and 8 μg of glucose-oxidase and 0.2 M sodium phosphate buffer (pH 7.0), made to 5 ml in a 50-ml flask, fitted with a one-hole rubber stopper containing a clamped capillary tube. The mixtures in flasks were incubated at 30° in a water-bath shaker and internal atmospheres above the reaction mixture were sampled, with a gas-tight syringe, after 10 min of incubation.

Gas analysis

Ethylene produced in the reaction was determined by gas chromatography using an alumina column and a flame-ionization detector. Details of the complete system have been described by MEIGH *et al.*[8].

Preparation of enzyme extracts

10 kg of apples were peeled and cored and then extracted twice with the same buffer solution (5 l), consisting of 0.015 M citrate–0.03 M sodium phosphate buffer (pH 7.2), containing $5 \cdot 10^{-3}$ M L-cysteine, 10^{-4} M Na_2SO_3, and 10^{-4} M EDTA, by maceration in a Waring Blendor for 2 min at top speed at 5°. The particles were then removed by centrifugation at 15 000 × *g* for 15 min. A total of about 10 l of supernatant fluid was collected.

Separation of phenols from extract

2-l aliquots of the supernatant fraction were added to a Sephadex G-25 column made with 500 g of Sephadex G-25, which was previously equilibrated with 0.015 M citric acid–0.03 M sodium phosphate (pH 7.2). The column was eluted with the same buffer, and collection was started with the first protein fraction eluting from the column.

After gel-filtration by Sephadex G-25, the protein solution was added to a DEAE-cellulose column, previously equilibrated with the same citrate–phosphate buffer. 2.5-l portions of the protein solution were applied to a column made up of 20 g DEAE-cellulose (column size, 7.5 cm × 5 cm). The column was washed with the same buffer, and the eluant from the column was collected with a fraction-collector. The enzymatic activity of each fraction was determined.

Separation from pectin-like substance

After chromatography on the DEAE-cellulose column, the pH of the protein solution was changed to 5.5 by dialyzing against 0.025 M citric acid–0.05 M sodium phosphate buffer (pH 5.5). 10 l of the protein solution was added to a column of 40 g CM-cellulose (column size, 7.5 cm × 7 cm) which was equilibrated with the same buffer. The enzyme was eluted with a linear NaCl concentration gradient, formed by allowing 500 ml of buffered 1.5 M NaCl to flow into 500 ml of the pH 5.5 equilibration buffer. 20-ml fractions were collected and assayed.

The enzyme fractions were combined (about 1.2 l) and dialyzed against the same buffer. The dialyzed enzyme fraction was chromatographed on 5 g of a CM-cellulose column (column size, 2.5 cm × 15 cm) with a linear gradient formed with 200 ml of the buffer and 200 ml of the buffered 1.5 M NaCl. 10-ml fractions were collected and assayed for activity.

Finally, after dialysis against the pH 5.5 buffer, about 300 ml of the enzyme fractions were added to 2 g of CM-cellulose column (column size, 1.3 cm × 13 cm) and rechromatographed with the pH 5.5 buffer containing a linear gradient of NaCl from 0 M to 1 M.

Electrophoresis

After CM-cellulose column chromatography, 70 ml of the enzyme fractions were collected and dialyzed against 0.033 M sodium phosphate buffer (pH 8.0). 20 g of dry Sephadex G-25 was added to 70 ml of the enzyme solution after the pH change, and allowed to swell for 30 min. 10 ml of the concentrated enzyme solution was separated from the Sephadex G-25 by centrifugation at 2000 rev./min for 10 min.

The concentrated enzyme solution was subjected to free-flow electrophoresis in the Brinkmann preparative electrophoresis system, Model FF-1. The buffers used were 0.033 M sodium phosphate buffer (pH 8.0), as the carrier buffer, and 0.1 M of the

same buffer, as the electrode rinsing buffer. Electrophoresis was carried out at 1500 V and 160 mA/50 cm^2. Sample injection rate was 2 ml/h.

Spectral analysis of the enzyme preparation

Spectral analysis in the ultraviolet region was carried out in the Perkin–Elmer Spectracord 4000 A. Spectral analysis in the visible region of the spectrum was carried out in a very sensitive spectrophotometer previously described[9]. The spectrophotometer was operated as a single beam instrument using a digital storage oscilloscope to provide system response correction for a flat baseline.

Determination of protein

The protein contents of the enzyme preparations were determined by ultraviolet absorption (A) at 280 nm (ref. 10), Nesslerization[11], and the LOWRY *et al.*[12] modification of the Folin method.

Assay of peroxidase activity

The rate of decomposition of H_2O_2 by peroxidase, with *o*-dianisidine as hydrogen donor, was determined by measuring the rate of color development at 460 nm for 2 min (ref. 13). The reaction mixture contained 6.0 ml of 0.003% H_2O_2 in 0.01 M phosphate buffer (pH 6.0), 0.05 ml of 1% *o*-dianisidine in methanol and 0.2 ml of enzyme solution.

RESULTS

Purification of 3-methylthiopropionaldehyde peroxidase

3-Methylthiopropionaldehyde peroxidase activity was confined almost exclusively (98%) to the supernatant fraction of both 15 000 × g (15 min) and 73 000 × g (90 min) centrifugations of the apple homogenate. MAPSON AND WARDALE[3] also reported that ethylene production by cauliflower extracts was confined to the supernatant fraction of a 20 000 × g centrifugation.

TABLE I

CHANGES IN SPECIFIC ACTIVITY DURING PURIFICATION

The enzyme activity was determined in the standard H_2O_2-generating system described in MATERIALS AND METHODS.

Stage of purification	*3-Methylthiopropionaldehyde peroxidase (µl ethylene per kg of apple)*	*Specific activity (µl of ethylene per mg of protein)*	*Total protein (mg/kg of apple)*
Enzyme extract (15 000 × g supernatant)	60	—	—
Enzyme extract after gel filtration with Sephadex G-25	160	0.6	260
Enzyme fraction after DEAE-cellulose column chromatography	200	—	—
Enzyme fraction after CM-cellulose column chromatography	158	3.2	49.5
Enzyme fraction after passing through CM-cellulose columns 3 times	51	204	0.25

A 2.5-fold increase in activity was obtained after gel filtration of the initial extract on Sephadex G-25 (Table I). Since the initial extract before gel filtration on Sephadex G-25 showed a blue color reaction with 1% $FeCl_2$ solution, a characteristic of phenols, and did not show the blue color reaction after Sephadex G-25 filtration, we suggest that the increase in activity resulted from separation of the enzyme from phenolic inhibitors present in the initial extract. The yellow color in the enzyme extract after gel filtration was mostly removed on the DEAE-cellulose column, but the enzyme was not adsorbed on this column at pH 7.0.

At this stage the enzyme fraction had a high viscosity due to its high pectin content, and thus formed a jelly-like substance upon addition of trichloroacetic acid. The pectin-like substances were separated from the enzyme by CM-cellulose chromatography. The pectins, which were not adsorbed on the column, exhibited virtually no 3-methylthiopropionaldehyde peroxidase activity. The enzyme adsorbed on CM-cellulose was subsequently eluted with a NaCl gradient and concentrated by passing through smaller CM-cellulose columns 3 times.

The enzyme fraction was then applied to the free-flow electrophoresis apparatus. The electrophoretic profile pattern of 3-methylthiopropionaldehyde peroxidase ob-

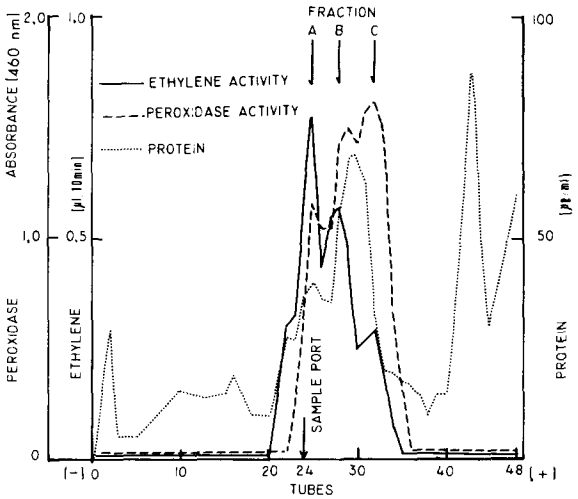
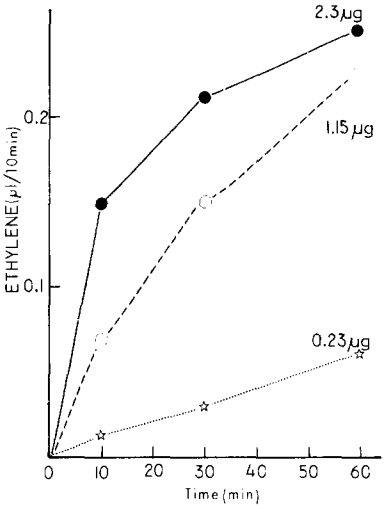


Fig. 1. Elution profile of ethylene formation, peroxidase activity and protein concentration after chromatography of the enzyme preparation by free-flow electrophoresis. Peroxidase activity refers to activity in the *o*-dianisidine reaction (see MATERIALS AND METHODS). Ethylene activity refers to 3-methylthiopropionaldehyde peroxidase activity.

tained at pH 8.2 (Fig. 1) suggests that the enzyme has three peaks of activity. No further purification of this enzyme was possible after electrophoresis because of the extremely low protein content and instability of the enzymatic activity. The enzyme lost activity rapidly at 0° (about 1/3 per day).

Characteristics of 3-methylthiopropionaldehyde peroxidase

Production of ethylene was proportional to enzyme concentration during the 10-min interval used in the enzyme assays (Fig. 2). The pH optima were 6.5 for the



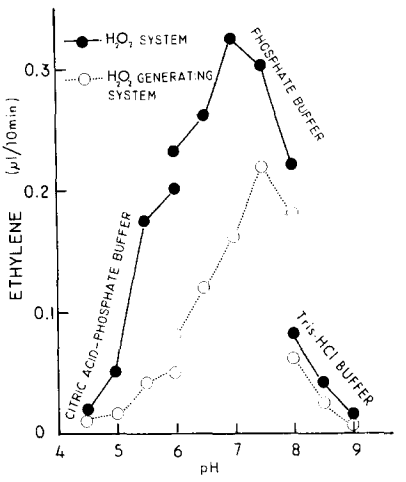


Fig. 2. Time-course of ethylene formation by 3-methylthiopropionaldehyde peroxidase. The reaction mixture contained, in a total volume of 5 ml, 10^{-3} M 3-methylthiopropionaldehyde, $2 \cdot 10^{-4}$ M *p*-coumaric acid, $2 \cdot 10^{-4}$ M $NaHSO_3$, $4 \cdot 10^{-4}$ M streptomycin, $7.6 \cdot 10^{-6}$ M H_2O_2, 0.2 M phosphate buffer (pH 7.0), and varied amounts of enzyme preparation as indicated.

Fig. 3. pH–Activity curve for ethylene formation. The components of the reaction in the H_2O_2 system are described in Fig. 2. In the H_2O_2-generating system glucose (1%) and glucose oxidase (8 μg) were substituted for H_2O_2.

H_2O_2-assay system and 7.0 for the glucose–glucose oxidase system (Fig. 3). Optimum concentration of H_2O_2 in the reaction was $7.6 \cdot 10^{-6}$ M (Fig. 4). Higher concentrations were inhibitory, due possibly to oxidation of the phenolic cofactor to its inactive quinone form.

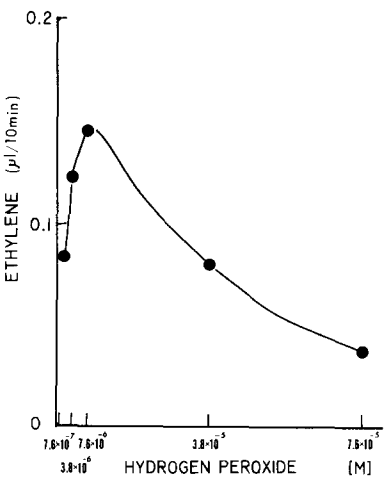


Fig. 4. Dependence of ethylene production on H_2O_2 concentration. Reaction mixtures were as described in Fig. 2.

TABLE II

COFACTOR REQUIREMENT FOR ETHYLENE PRODUCTION BY THE 3-METHYLTHIOPROPIONALDEHYDE PEROXIDASE SYSTEM

The complete reaction mixture contained in a total volume of 5 ml, 10^{-3} M 3-methylthiopropionaldehyde, $2 \cdot 10^{-4}$ M $NaHSO_3$, $2 \cdot 10^{-4}$ M *p*-coumaric acid, $7.6 \cdot 10^{-6}$ M H_2O_2, 0.2 ml of enzyme preparation and 3 ml of 0.2 M phosphate buffer (pH 7.0).

Component	*Ethylene production (nl/10 min)*
Complete	270
Enzyme omitted	20
3-Methylthiopropionaldehyde omitted	0
p-Coumaric acid omitted	10
SO_3^{2-} omitted	0
H_2O_2 omitted	0
Complete but with boiled enzyme	10

TABLE III

ETHYLENE PRODUCTION BY 3-METHYLTHIOPROPIONALDEHYDE PEROXIDASE AND HORSERADISH PEROXIDASE SYSTEMS

The reaction mixture of the H_2O_2-generating system was as in Table II, except that 8 μg of glucose-oxidase and 1% glucose were substituted for H_2O_2. The reaction mixture of the Mn^{2+} system was as in Table II except the $2.5 \cdot 10^{-4}$ M $MnSO_4$ substituted for H_2O_2. Horseradish peroxidase concentration was 0.2 μg/ml. Resorcinol ($2 \cdot 10^{-5}$ M) was used as the electron donor in the horseradish peroxidase reaction.

System	*3-Methylthiopropionaldehyde peroxidase (nl/10 min)*	*Horseradish peroxidase (nl/10 min)*
H_2O_2-generating	150	500
Mn^{2+} + $2 \cdot 10^{-4}$ M *p*-coumaric acid	2	100
Mn^{2+} + $2 \cdot 10^{-5}$ M *p*-coumaric acid	16	1050

Cofactors

The 3-methylthiopropionaldehyde peroxidase system from apples shows a requirement for $2 \cdot 10^{-4}$ M *p*-coumaric acid and for SO_3^{2-} in addition to H_2O_2 (Table II), much the same as the cauliflower enzyme[5]. The H_2O_2 requirement of 3-methylthiopropionaldehyde peroxidase cannot be replaced by Mn^{2+}, a potent catalyst in the horseradish peroxidase 3-methylthiopropionaldehyde-cleavage system (Table III).

The cofactor suitability of several phenols in the 3-methylthiopropionaldehyde peroxidase system and in the horseradish peroxidase 3-methylthiopropionaldehyde-cleavage system is shown in Table IV. Cofactor activity in 3-methylthiopropionaldehyde peroxidase was shown only by $2 \cdot 10^{-4}$ M *p*-coumaric acid or its methyl ester, whereas *p*-hydroxy benzoic acid, which is very active in the cauliflower enzyme system and with horseradish peroxidase, and other related phenolic substances, including a lower concentration of *p*-coumaric acid, were inactive with the apple enzyme.

TABLE IV

COFACTOR SUITABILITY OF PHENOLS IN 3-METHYLTHIOPROPIONALDEHYDE-CLEAVAGE REACTION

The reaction mixtures were as in Table III except that *p*-coumaric acid was substituted by other phenols. Absolute values of ethylene production are shown in Table III.

Component	*Concn. (M)*	*Activity**			
		3-Methylthiopropionaldehyde peroxidase		*Horseradish peroxidase*	
		H_2O_2	Mn^{2+}	H_2O_2	Mn^{2+}
p-Coumaric acid	$2 \cdot 10^{-4}$	100	1	100	17
	$2 \cdot 10^{-5}$	15	10	25	200
m-Coumaric acid	$2 \cdot 10^{-4}$	13	—	—	—
o-Coumaric acid	$2 \cdot 10^{-4}$	15	—	—	—
p-Coumaric acid methyl ester	$2 \cdot 10^{-4}$	100	—	—	—
p-Hydroxy benzoic acid	$2 \cdot 10^{-4}$	3	5	60	100
	$2 \cdot 10^{-5}$	0	1	6	39
Caffeic acid	$2 \cdot 10^{-4}$	10	—	—	—
Cinnamic acid	$2 \cdot 10^{-4}$	0	—	—	—
2,4-Dichlorophenol	$2 \cdot 10^{-4}$	0	0	2	30
	$2 \cdot 10^{-5}$	0	0	1	20
Resorcinol	$2 \cdot 10^{-4}$	5	—	—	—
	$2 \cdot 10^{-5}$	0	0	20	80
Thyroxine	$2.7 \cdot 10^{-5}$	10	—	13	—

* As percent of rate of ethylene formation with $2 \cdot 10^{-4}$ M *p*-coumaric acid.

Inhibitors

3-Methylthiopropionaldehyde peroxidase is effectively inhibited by metal chelating agents such as sodium diethyldithiocarbamate, cupferron, and metal-enzyme inhibitors like NaN_3, and also by SH-group inhibitors such as iodoacetamide and *N*-ethylmaleimide. EDTA is considerably less inhibitory than the other metal chelating agents, and was found to stimulate the reaction a little at a concentration of $2 \cdot 10^{-4}$ M. Catalase (1 μg), ascorbate, and low concentrations of Cu^{2+} and (+)-catechin strongly inhibited the system (Table V). These inhibitor characteristics are very similar to those reported for the cauliflower 3-methylthiopropionaldehyde-cleavage reaetion and for the horseradish peroxidase 3-methylthiopropionaldehyde-cleavage system[5,6].

Streptomycin sulfate as activator

Streptomycin sulfate ($9 \cdot 10^{-4}$ M), which was used to preserve the extract during purification, more than doubled 3-methylthiopropionaldehyde peroxidase activity (Table VI). This effect, but to a lesser extent, was also observed in the 3-methylthiopropionaldehyde-cleavage activity of horseradish peroxidase. On the other hand, streptomycin inhibited ethylene production from 3-methylthiopropionaldehyde in the Cu^{2+}–ascorbate model system and also inhibited catalase activity (Table VI). Streptomycin activation of the 3-methylthiopropionaldehyde peroxidase system may result from chelation of copper ions[14], which competitively inhibit free radical intermediates, or from inhibitory degradation of H_2O_2 needed in the reaction.

TABLE V

EFFECT OF INHIBITORS ON 3-METHYLTHIOPROPIANALDEHYDE PEROXIDASE

The reaction mixtures were as in Table IV. The inhibition tests with Cu^{2+} and (+)-catechin were examined in the H_2O_2 system with streptomycin ($9 \cdot 10^{-4}$ M) .

Inhibitor	*Concn. (M)*	*Inhibition (%)*
EDTA	$2 \cdot 10^{-4}$	−11*
	$2 \cdot 10^{-3}$	16
Sodium diethyldithiocarbamate	10^{-3}	100
Cupferron	10^{-3}	100
NaN_3	$5 \cdot 10^{-3}$	34
Iodoacetamide	10^{-3}	30
N-Ethylmaleimide	$3 \cdot 10^{-6}$	0
	$3 \cdot 10^{-5}$	7
	$3 \cdot 10^{-4}$	16
	$3 \cdot 10^{-3}$	52
Catalase	0.2 μg/ml	80
	0.02 μg/ml	10
Ascorbate	10^{-2}	80
Cu^{2+}	10^{-6}	70
	10^{-5}	82
(+)-Catechin	10^{-6}	94
	10^{-5}	100

* Negative sign indicates activation.

Comparison of o-dianisidine and 3-methylthiopropionaldehyde peroxidase activity in fractions separated by free-flow electrophoresis

The three enzyme fractions, A, B and C, separated by free-flow electrophoresis, showed different arbitrary ratios of *o*-dianisidine (peroxidase) to 3-methylthiopropionaldehyde-cleavage activity (Table VII). These arbitrary ratios were also different

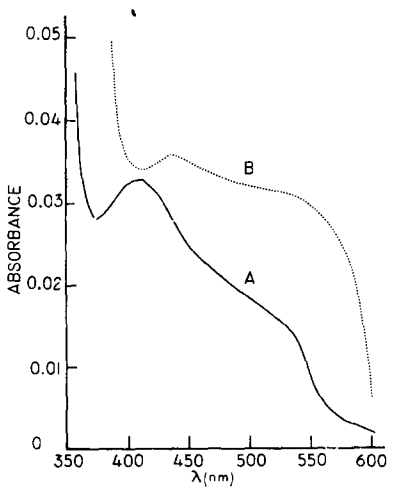


Fig. 5. Change in the visible absorption spectrum of purified 3-methylthiopropionaldehyde peroxidase with a few crystals of sodium dithionite. Curve A, no dithionite; Curve B, dithionite. Protein concentration was 23 μg/ml.

TABLE VI

EFFECT OF STREPTOMYCIN ON THE 3-METHYLTHIOPROPIONALDEHYDE PEROXIDASE AND HORSERADISH PEROXIDASE SYSTEMS, THE Cu^{2+}–ASCORBATE MODEL SYSTEM, AND CATALASE

The reaction mixtures for the 3-methylthiopropionaldehyde peroxidase and horseradish peroxidase systems were the same as in Tables II and III. The Cu^{2+}–ascorbate model system contained 10^{-4} M Cu^{2+}, 10^{-3} M 3-methylthiopropionaldehyde, 10^{-3} M ascorbate in 0.2 M phosphate buffer (pH 7.0), in a total volume of 5 ml. Catalase was assayed in two steps. The first step involved H_2O_2 degradation in the following system: 1 ml H_2O_2 (3%), 0.2 ml catalase (0.1 mg/ml) *plus* or *minus* 1 ml streptomycin sulfate ($9 \cdot 10^{-4}$ M) in 0.2 M phosphate buffer (pH 7.0), incubated for 5 min at 30°. The next step involved determination of H_2O_2 in the horseradish peroxidase assay system as follows: 2 ml benzidine (0.1%), 0.1 ml horseradish peroxidase (1 mg/ml) and 2 ml phosphate buffer, added to the H_2O_2 degradation system. The optical absorption of the mixture at 460 nm was determined after 10 min of incubation at 30°. A control with no catalase and no streptomycin, to determine spontaneous breakdown of H_2O_2, was assayed similarly.

System	*Streptomycin concn.* $\times 10^4$ *(M)*	*Ethylene production*	*Change (%)**
		μl/10 ml per 10 min	
3-Methylthiopropionaldehyde peroxidase	0	1.2	—
	4.5	2.0	+66
	9.0	2.9	+140
		μl/μg per 10 min	
Horseradish peroxidase	0	0.4	—
	1.0	0.4	0
	4.5	0.5	+25
	9.0	0.7	+75
		μl/10 min	
Cu^{2+}–ascorbate model	0	0.5	—
	9.0	0.1	−80
Cu^{2+}–ascorbate model + sodium diethyldithiocarbamate (10^{-4} M)	0	0.3	−40
	9.0	0.1	−80
Catalase	0	—	—
	9.0	—	−59

* Negative signs indicate inhibition, positive ones activation.

from the ratios obtained with commercial horseradish peroxidase. Fraction A had the greatest activity and Fraction C had the lowest activity with 3-methylthiopropionaldehyde; Fraction C had a ratio of *o*-dianisidine (peroxidase) activity to 3-methylthiopropionaldehyde cleavage which was similar to that of horseradish peroxidase. Purified 3-methylthiopropionaldehyde peroxidase thus appears to have some catalytic characteristics resembling horseradish peroxidase and others which differ from it.

Absorption spectra of the 3-methylthiopropionaldehyde peroxidase preparation

The 3-methylthiopropionaldehyde peroxidase preparation, purified by CM-cellulose 3 times, showed maximum absorption at 278 nm. No absorption was obtained in the visible region of the spectrum with an ordinary spectrophotometer. However, at very high resolution in the multispectrophotometer[9] the enzyme absorbed maximally near the 410-nm region, with a shoulder in the 525-nm region, as shown in Fig. 5. It is

TABLE VII

ACTIVITY OF THREE 3-METHYLTHIOPROPIONALDEHYDE PEROXIDASE FRACTIONS SEPARATED BY FREE-FLOW ELECTROPHORESIS IN THE *o*-DIANISIDINE (HORSERADISH PEROXIDASE) AND 3-METHYLTHIOPROPIONALDEHYDE REACTION

Activity of peroxidase was determined by the *o*-dianisidine reaction system[13]. The standard ethylene-forming reaction system described in MATERIALS AND METHODS was used to determine 3-methylthiopropionaldehyde peroxidase activity.

Fraction	*o-Dianisidine reaction ($\Delta A_{460\ nm}$/min per mg protein)*	*Ethylene production (μl/10 min per mg protein)*	*Ratio of o-dianisidine reaction to ethylene-producing reaction*
A	29.3	18.8	1.6
B	26.1	10.3	2.5
C	52.8	6.6	8.0
Horseradish peroxidase	4480.0	700.0	6.4

possible that the absorption near 410 nm represents a Soret band because it shifted to about 440 nm on addition of dithionite. Horseradish peroxidase behaves similarly upon addition of dithionite[15]. If the broad absorption band shown by the 3-methylthiopropionaldehyde peroxidase preparation near 410 nm is a Soret band, it could have been altered or otherwise interfered with by impurities in the preparation.Since a solution of commercial horseradish peroxidase with about the same protein content (30 μg/ml) gives a much larger and sharper band, it is likely that this enzyme is not an horseradish peroxidase-type enzyme. It is also possible that 3-methylthiopropionaldehyde peroxidase is not a typical heme enzyme.

Ethylene production from L-*methionine activated by 3-methylthiopropionaldehyde peroxidase*

The Cu^{2+}–ascorbate model system produces ethylene from L-methionine[1]. It is also known that horseradish peroxidase produces aldehydes from some amino acids in a Mn^{2+}–peroxidase system containing codecarboxylase[16]. It is probable that 3-methylthiopropionaldehyde, which is a precursor of ethylene, is formed from L-methionine *in vivo*.

3-Methylthiopropionaldehyde peroxidase produced ethylene from L-methionine by addition of codecarboxylase, but activity of the methionine-cleavage reaction was only about 20% of the 3-methylthiopropionaldehyde-cleavage reaction (Table VIII).

The methionine-cleavage reaction was separated into two steps. In the first step 3-methylthiopropionaldehyde peroxidase produces intermediates by reacting with Mn^{2+}, *p*-coumaric acid, codecarboxylase and methionine. In the second step, ethylene is produced from the intermediates by addition of HSO_3^- and the H_2O_2-generating system. If HSO_3^- and the H_2O_2-generating system are added to the reaction mixture at the beginning of the reaction, ethylene production is depressed.

From these results it is assumed that ethylene production from L-methionine *in vivo* may require two different systems which are separated in the cell, since they appear to be antagonistic.

TABLE VIII

ETHYLENE PRODUCTION FROM L-METHIONINE AND 3-METHYLTHIOPROPIONALDEHYDE BY 3-METHYLTHIOPROPIONALDEHYDE PEROXIDASE PREPARATION

Expt. I. Reaction mixture contained 0.5 ml of enzyme preparation (protein 20 μg/ml), 10^{-3} M of L-methionine, $2.5 \cdot 10^{-4}$ M $MnSO_4$, $2 \cdot 10^{-4}$ M *p*-coumaric acid and 10^{-3} M codecarboxylase. Reaction mixture was incubated for 60 min at 30°. *Expt. II.* After 60 min of incubation under the above conditions, $2 \cdot 10^{-4}$ M $NaHSO_3$, 1% glucose and 8 μg glucose oxidase were added in the system of *Expt. I.* Incubation period, 10 min at 30°. *Expt. III.* All reagents were mixed at the beginning and incubated for 10 min at 30°. *Expt. IV.* 3-Methylthiopropionaldehyde-cleavage standard system with no streptomycin. Incubation period, 10 min at 30°.

Expt. No.	*Ethylene production (μl/10 ml of enzyme preparation)*
I	0.24
II	3.72
III	0.24
IV	16.80

DISCUSSION

Although apple slices produce large quantities of ethylene, apple homogenates show virtually no ethylene formation[8]. However, after separation of phenols by gel filtration with Sephadex G-25, we demonstrated the presence of an ethylene-forming system in apple homogenates. The activity of this ethylene-forming system (3-methylthiopropionaldehyde peroxidase) was inhibited by diphenols, such as catechin and chlorogenic acid, which are known to be present in apples[17,18].

The 3-methylthiopropionaldehyde peroxidase system from apple requires a phenol, HSO_3^-, and H_2O_2, much the same as the horseradish peroxidase 3-methylthiopropionaldehyde-cleavage system[6] and the 3-methylthiopropionaldehyde-cleavage enzyme from cauliflower[3-5]. However, unlike the horseradish peroxidase system Mn^{2+} cannot replace H_2O_2 in the 3-methylthiopropionaldehyde peroxidase reaction, and like the cauliflower enzyme a monophenol is required for activity. 3-Methylthiopropionaldehyde peroxidase from apple specifically requires *p*-coumaric acid or its methyl ester, and cannot operate with *p*-hydroxybenzoic acid which is active with the cauliflower enzyme. With both 3-methylthiopropionaldehyde-cleavage enzymes and the horseradish peroxidase system only a *p*-phenol is operative, while an *o*- or *m*-phenol is virtually inactive.

3-Methylthiopropionaldehyde peroxidase from apples is also inhibited by most of the same agents (catalase, sodium diethyldithiocarbamate, Cu^{2+}) which inhibit the horseradish peroxidase system or the cauliflower 3-methylthiopropionaldehyde-cleavage system. Though each of these enzymes seems to have a few different characteristics, the basic mechanism of the 3-methylthiopropionaldehyde-cleavage reaction appears similar, as indicated by the requirements of the systems which suggest the involvement of free radicals. There is also some similarity in the spectral absorption pattern shown by 3-methylpropionaldehyde peroxidase and horseradish peroxidase.

Therefore we believe that these three enzymes belong to the same family of peroxidase-cleavage enzymes.

3-Methylthiopropionaldehyde peroxidase can also produce a small amount of ethylene from L-methionine, in a two-step reaction in which the first step is similar to the Mn^{2+}–codecarboxylase peroxidatic reaction which converts some amino acids to aldehydes[16]. The second step is the 3-methylthiopropionaldehyde-cleavage reaction. Because ethylene production is depressed or virtually eliminated, *in vitro*, when the two systems are combined initially, we suggest that ethylene production from L-methionine *in vivo* is dependent on two reaction systems which are separated within the cell. The first system may be mediated by Mn^{2+} and produces 3-methylthiopropionaldehyde, and the second system requires H_2O_2 and a free radical, such as sulfite ion, to cleave 3-methylthiopropionaldehyde. If the same enzyme is involved in both reactions then it is possible that the cofactors Mn^{2+} and H_2O_2 alter the protein to change its reactivity characteristics.

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