FORMATION OF 3-METHYLTHIOACRYLIC ACID FROM METHIONINE BY *STREPTOMYCES LINCOLNENSIS*

ISOLATION OF A PEROXIDASE

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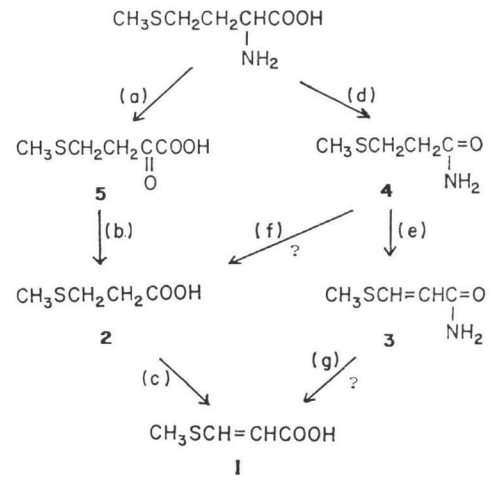
Cultures of *Streptomyces lincolnensis* accumulated 3-methylthioacrylic acid in amounts directly related to the concentration of methionine in the medium. The metabolite was labeled by L-(*methyl*-^{14}C) but not by DL-(*carboxyl*-^{14}C) methionine, indicating biosynthesis from the amino acid with loss of the carboxyl group. *S. lincolnensis* mycelium contained sufficient peroxidase activity to catalyse oxidative decarboxylation of L-methionine to 3-methylthiopropionamide as the initial step of a biosynthetic sequence. The enzyme, partially purified by ammonium sulfate precipitation, chromatography on a DEAE-cellulose column and gel filtration, had a molecular weight of approximately 350,000, a pH optimum of 6.0, with *o*-dianisidine as electron donor and a K_m value of 7.5×10^{-4} M with respect to hydrogen peroxide. Cultures of *S. lincolnensis* supplemented with 3-(*methyl*-^{14}C) methylthiopropionic acid gave labeled 3-methylthioacrylic acid. However, 3-(*methyl*-^{14}C) methylthiopropionamide did not label the metabolite, suggesting that the first intermediate in the pathway may be the keto acid, which is then oxidatively decarboxylated to 3-methylthiopropionic acid.

3-Methylthioacrylic acid (**1**) was first described as a product of microbial fermentation by VISSER and MEYER[1] who isolated it from lincomycin-producing cultures of a *Streptomyces lincolnensis* mutant that had been grown in media supplemented with methionine. Independently ARIMA and co-workers[2] identified it, and 3-methylthiopropionic acid (**2**) as the active substances in *Streptomyces* species 340 that caused a behavioural change in planaria. Production of the metabolites was associated with methionine in the nutrient medium, and was widely distributed among *Streptomyces* species. Smaller amounts were produced by some fungi, but none could be detected in bacterial or yeast cultures. ARIMA and his colleagues concluded that the two products were formed from methionine and suggested the route $a \rightarrow b \rightarrow c$ (Scheme 1). They reported preliminary evidence for step *c*.

Scheme 1. Possible biosynthetic pathways leading to 3-methylthioacrylic acid.



YAGI and co-workers[3] have since isolated the amide (**3**) of 3-methylthioacrylic acid from cultures of the siomycin-producing *Streptomyces sioyaensis*, and the same compound, accompanied by 3-methylthiopropionamide (**4**), was recently isolated from puromycin-producing cultures of *Streptomyces alboniger* by FROHWEIN *et al.*[4] Again, formation of the metabolites was

associated with methionine in the nutrient medium, and FROHWEIN and his colleagues noted that peroxidase can catalyze the oxidative decarboxylation of methionine to 3-methylthiopropionamide.[5,6] They suggested this as a likely route *in vivo*, with 3-methylthioacrylamide being formed by subsequent dehydrogenation (steps *d* and *e*, Scheme 1).

It is apparent that extension of this latter pathway by the action of an amidase (steps *f* and *g*, Scheme 1) could also account for the formation of 3-methylthiopropionic and 3-methylthioacrylic acids in some organisms, the precise route and balance of products depending upon the specificities and relative activities of the required amidases and dehydrogenases. A possible argument against this hypothesis was the absence from the literature of any report of peroxidase activity in the Actinomycetales. Our objectives in the present study were (i) to verify that 3-methylthioacrylic acid in cultures of *S. lincolnensis* is formed specifically from methionine with loss of the carboxyl group, (ii) to determine whether peroxidase was present in *S. lincolnensis* in amounts sufficient to account for the conversion, and (iii) to examine whether potential intermediates in the proposed pathways were metabolized *in vivo* to 3-methylthioacrylic acid.

Materials and Methods

Culture

Streptomyces lincolnensis NRRL 2936 was obtained from the Northern Regional Research Laboratory of the United States Department of Agriculture, Peoria, Illinois. It was maintained on yeast extract agar.[7] A vegetative inoculum was prepared by incubating spores and mycelium from the slant for 48 hours in 250-ml Erlenmeyer flasks containing 50 ml of a D-glucose (1%), yeast extract (1%), N-Z amine type B (0.5%) solution in distilled water. Cultures for the production of 3-methylthioacrylic acid were grown from a 5% (v/v) vegetative inoculum in 1-liter Erlenmeyer flasks containing 500 ml of the following medium: D-glucose (2%), soybean meal (1%), calcium carbonate (0.4%), DL-methionine (0.5%) in distilled water. Vegetative inoculum and production cultures were incubated at 26°C on a shaker with a platform rotating at 220 rpm in a 3.8 cm circle.

Chemicals

Yeast extract was obtained from Difco Laboratories, Detroit, Michigan; N-Z amine type B from Sheffield Chemicals, Norwich, New York; soybean meal from Canada Packers Limited, Toronto, Ontario; *o*-dianisidine from the British Drug House Limited, Toronto, Ontario; 3-mercaptopropionic acid from Eastman Organic Chemicals, Rochester, New York. Authentic specimens of 3-methylthioacrylic acid and 3-methylthiopropionamide were gifts from Dr. J. VISSER., The Upjohn Company, Kalamazoo, Michigan, and Dr. M. MAZELIS, Department of Food Science and Technology, University of California, Davis, California, respectively.

(^{14}C) Dimethyl sulfate, DL-(*carboxyl*-^{14}C) methionine and L-(*methyl*-^{14}C) methionine were purchased from New England Nuclear Corporation, Boston, Massachusetts. 3-(*Methyl*-^{14}C) methylthiopropionic acid was prepared by treating 3-mercaptopropionic acid with (^{14}C) dimethyl sulfate.[8] To obtain 3-(*methyl*-^{14}C) methylthiopropionamide, the acid was first esterified with diazomethane and then treated with excess methanolic ammonia. The product was recrystallized to radiochemical purity from a mixture of ethyl acetate and petroleum ether (b.p. 60~80°C).

Estimation of 3-Methylthioacrylic Acid

Culture filtrates were acidified, extracted with ethyl acetate, and the extract assayed for compounds catalyzing the azide-iodide reaction. The procedure used was similar to that described by RUSSELL[9] and was calibrated with 3-methylthioacrylic acid.

Thin-Layer Chromatography

Silica gel HF_{254} (E Merck, Darmstadt) was used as adsorbent with either: A, *n*-butanol - 1.5 N ammonium hydroxide (1: 1); B, benzene - acetic acid - water (42: 24: 1) or C, benzene - petroleum ether (b.p. 30~80°C) - acetic acid - water (5: 3: 5: 3), as solvents. 3-Methylthioacrylic acid (R_f: A, 0.28; B, 0.63; C, 0.29) was readily detected by fluorescence quenching at 254 nm; 3-methylthiopro-

pionic acid (R_f: A, 0.26; B, 0.56; C, 0.26) and 3-methylthiopropionamide (R_f: A, 0.62; B, 0.43; C, 0.08) as well as the unsaturated acid were detected at low concentrations by spraying plates with the azide-iodide reagent.[10]

Isolation of 3-Methylthioacrylic Acid

The procedure was modified from that of VISSER and MEYER.[1] The mycelium was removed by centrifugation and the culture supernatant at pH 2.5 was extracted with ethyl acetate. Acidic products in the extract were back-extracted into water at pH 10, freed from non-acidic material by washing with methylene dichloride, and recovered after acidification by transferring to ethyl acetate. Crude 3-methylthioacrylic acid was purified either directly by crystallization from aqueous acetone, then a mixture of ethyl acetate and petroleum ether (b.p. 60~80°C), or by chromatography on a column of silicic acid with a stepped gradient of chloroform in benzene. 3-Methylthioacrylic acid was eluted at a concentration of 20% chloroform. The purified compound, m.p. 139°C, was identified by comparison with an authentic specimen and its radiochemical purity was verified by thin-layer chromatography.

Assay for Peroxidase

For routine assays during enzyme purification the colorimetric procedure described in the Worthington Enzyme Manual[11] was used. The reaction mixture contained: *o*-dianisidine (1 μmole), hydrogen peroxide (2.6 μmoles), sodium phosphate buffer, pH 7.0 (2.9 μmoles) and enzyme solution (0.1 ml) in a total volume of 3 ml water. It was incubated at 25°C and peroxidase activity was measured as the initial rate of increase in absorbance at 460 nm. A unit of activity was defined as the amount of enzyme giving an absorbance increase of 1.0 after 10 minutes. Under the conditions used, 1 μg of Sigma Type VI horseradish peroxidase (310 purpurogallin 20-second units) contained 59 units of activity.

Samples were also assayed for ability to catalyze the release of (^{14}C) carbon dioxide from labeled methionine. The assay mixture (total volume, 2 ml) contained: sodium phosphate buffer, pH 7.0 (100 μmoles); DL-(*carboxyl*-^{14}C) methionine (2 μmoles, 0.5 μCi); manganous chloride (0.5 μmole) pyridoxal phosphate (0.5 ml of a 2 mM-solution in 0.2 M sodium phosphate buffer, pH 7.0); enzyme solution (0.5 ml). The reaction was started by adding methionine as the last ingredient, and after 1 hour at 30°C it was stopped by adding 6 N hydrochloric acid (0.3 ml). (^{14}C) Carbon dioxide was swept from the reaction mixture and trapped in sodium hydroxide solution. Enzyme activity, estimated as nano moles of carbon dioxide produced, under the conditions of the assay, was determined from the amount of radioactivity trapped, after subtracting the amount obtained in a blank determination from which enzyme was omitted.

Estimation of Protein

In crude extracts and during early purification steps, protein was estimated by the biuret reaction.[12] During and after fractionation of enzyme samples with DEAE-cellulose, protein was estimated from extinction measurements at 260 and 280 nm using the nomograph of WARBURG and CHRISTIAN.[13]

Isolation of Peroxidase

Streptomyces lincolnensis was grown for 2 days under the usual conditions for producing 3-methylthioacrylic acid. The mycelium from 4 liters of broth was collected by centrifugation. This and all subsequent operations were carried out at 4°C. The cells were resuspended in 0.1 M sodium phosphate buffer, pH 7.0 (150 ml), disrupted by sonic oscillation for 10 minutes, and centrifuged (25,000 × *g*, 30 minutes). Ammonium sulfate was added to the supernatant solution and the precipitate collected at 40~60% saturation was redissolved in 0.1 M sodium phosphate buffer, pH 7.0 (20 ml). The solution, dialyzed for 6 hours against the same buffer, was applied to a column (2.5 × 45 cm) of DEAE-cellulose developed first with 600 ml of the buffer alone, then with 600 ml of the buffer containing 0.2 M potassium chloride, and finally with the buffer containing a 0.2~0.4 M potassium chloride gradient. Fractions (5 ml) were collected after beginning the salt gradient and assayed for protein content and enzyme activity. Those with peroxidase activity (fractions 11~16) were combined, reduced in volume, and the buffer replaced with 1 mM sodium phosphate, pH 7.0, by ultrafiltration (Diaflo UM 10 membrane, Amicon Corporation). The samples (5 ml) were applied to a column

(2.5×65 cm) of cross-linked dextran gel (Sephadex G-200, Pharmacia). The column was developed with 1 mM sodium phosphate buffer, pH 7.0 and 5 ml fractions were collected.

Results

Effect of Methionine

Cultures of *S. lincolnensis* grown in glucose-soybean meal medium supplemented with DL-methionine produced 3-methylthioacrylic acid in increasing amount up to the fourth day (Fig. 1)*. The yield of the metabolite in culture supernatant solutions depended upon the amount of methionine present in the medium (Table 1).

Incorporation of ^{14}C-Labeled Compounds

When L-(*methyl*-^{14}C) methionine was added to the culture medium, radioactive 3-methylthioacrylic acid was formed with a moderate dilution in specific activity (Table 2). In contrast, the metabolite isolated from cultures supplemented with DL-(*carboxyl*-^{14}C) methionine was radio-inactive. Of the two potential intermediates in the metabolism of methionine to 3-methylthioacrylic acid that were tested, only 3-(*methyl*-^{14}C) methylthiopropionic acid labeled the product. No radioactivity was incorporated from 3-(*methyl*-^{14}C) methylthiopropionamide.

Isolation of Peroxidase

Disruption of the mycelium from cultures of *S. lincolnensis* grown in a medium containing DL-methionine and harvested when 3-methylthioacrylic acid was being produced gave a cell homogenate with weak peroxidase activity in the colorimetric assay using *o*-dianisidine as electron donor (Table 3). The activity remained in the supernatant fraction upon centrifugation and could be precipitated with ammonium sulfate. The protein fraction precipitated at 40~60% saturation had the highest specific activity and further purification was achieved by chromatography on a column of DEAE-cellulose. Activity was retained on the column when the sample was applied in 0.1 M sodium phosphate buffer, pH 7.0, and was eluted at a potassium chloride concentration of approximately 0.3 M. For routine purification of the enzyme, the column was washed, after the sample had been applied, with buffer containing 0.2 M potassium chloride to

Fig. 1. Production of 3-methylthioacrylic acid in cultures of *S. lincolnensis*.

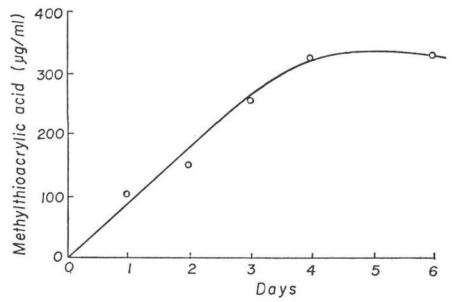


Table 1. 3-Methylthioacrylic acid in cultures grown on media with different methionine contents.

Methionine isomer	g/liter	3-Methylthioacrylic acid (mg/liter)
L	0.5	114
L	1.0	151
L	2.0	271
L	3.0	277
L	5.0	373
DL	2.0	280

The basal medium contained D-glucose (2%) soybean meal (1%) and calcium carbonate (0.4%) adjusted to pH 7.2. Cultures were grown for 4 days and assayed for 3-methylthioacrylic acid by the azide-iodide procedure.

* An apparent difference between the rate of production by this strain and by the mutant strain of VISSER and MEYER[1] is due to an error in Figure 1 of their paper, where the abscissa should read days instead of hours (J. VISSER, personal communication).

Table 2. Incorporation of radioactivity into 3-methylthioacrylic acid from ^{14}C-labeled substrates.

Labeled substrate	nC/mmole	3-Methylthioacrylic acid	
		nC/mmole	%*
L-(*methyl*-^{14}C) Methionine	149	29.0	19.6
DL-(*carboxyl*-^{14}C) Methionine	149	Nil	—
3-(*methyl*-^{14}C) Methylthiopropionic acid	2,470	80.0	3.2
3-(*methyl*-^{14}C) Methylthiopropionamide	2,470	Nil	—

* Incorporation measured as 100 × specific activity of product ÷ specific activity of substrate.

Table 3. Purification of peroxidase from *S. lincolnensis*.

Fraction	Volume (ml)	Protein (mg/ml)	Peroxidase activity			
			units/ml	units/ mg protein	% Recovery	Purification
Crude extract	70	28.2	1.70	0.06	100	0
$(NH_4)_2SO_4$ precipitate 40~60%	6.1	20.4	184	9.0	935	150
DEAE-cellulose fractions	5.0	0.96	24.7	25.7	103	428
Sephadex G-200 fractions	4.0	0.32	11.5	35.9	35.6	597

Table 4. Peroxidative decarboxylation of DL-(*carboxyl*-^{14}C) methionine*

Enzyme sample		Yield of $^{14}CO_2$ n moles
Horseradish peroxidase	11.8 units**	502
S. lincolnensis peroxidase crude enzyme	11.8	0
after DEAE-cellulose chromatography	0.18	2.9
after Sephadex chromatography	2.35	19.8
Mixture: horseradish peroxidase	7.1	0
S. lincolnensis crude enzyme	4.7	

* Enzyme samples were incubated with the labeled amino acid and the radioactive carbon dioxide evolved was measured as described in the text.

** Activity was measured by the colorimetric assay using *o*-dianisidine as electron donor.

remove inactive proteins, and peroxidase was then eluted with a shallow potassium chloride gradient. The active fractions from this column were rechromatographed on a column of Sephadex G-200 gel and gave a single peak of peroxidase activity. Enzyme recovery and the specific activities of fractions obtained during the purification are summarized in Table 3.

Properties of Peroxidase

Samples of the enzyme obtained from DEAE-cellulose columns showed a pH optimum in 0.1 M phosphate and 0.1 M phthalate buffers of 6.0. The enzyme was relatively stable and could be stored for two weeks at 4°C with only 25% loss and at −20° with no detectable loss in activity. At 37°C activity was reduced by less than 10% after 10 minutes but at 60°C for 2 minutes all activity was lost. The enzyme showed an absolute requirement for hydrogen peroxide and the K_m value for this substrate was 7.5×10^{-4} M. The molecular weight estimated by gel-filtration through a column of Sephadex

G-200 as described by ANDREWS[14] was 350,000 (± 60,000). Samples of crude enzyme or fractions obtained by ammonium sulfate precipitation showed no activity when assayed for their ability to catalyze the oxidative decarboxylation of DL-(*carboxyl*-^{14}C) methionine. After further purification by DEAE-cellulose chromatography, they became active and their requirements were similar to those reported for horseradish peroxidase (Table 4).

Discussion

From the increased yield of 3-methylthioacrylic acid when methionine is added to the culture, and from the efficient incorporation of isotope from methionine labeled in the methyl group but failure to incorporate the carboxyl group, it is evident that the metabolite is formed by a relatively direct route from this amino acid. We did not compare the precursor efficiency of the enantiomers but the L- and DL-forms were equally effective in increasing the yield of 3-methylthioacrylic acid in cultures. ARIMA *et al.*[2] observed that both D-and L-isomers promoted some increase of yield in *Streptomyces fradiae* cultures but the latter was much more effective. The racemate gave almost as high a yield as the L-isomer. In the absence of information about the interconversion of epimers of methionine and its corresponding α-keto acid these results do not distinguish between L-methionine and 4-methylthio-2-ketobutyric acid (**5**) as the more immediate precursor of 3-methylthioacrylic acid.

Information published by MAZELIS[5] on the rate at which horseradish peroxidase catalyzes the oxidative decarboxylation of L-methionine under optimal conditions suggests that activity equivalent to at least 0.26 μg of horseradish peroxidase should be present in 1 liter of *S. lincolnensis* culture to account for the observed rate of 3-methylthioacrylic acid accumulation. Such low activity cannot be detected in disrupted cell suspensions by the direct decarboxylation assay using (*carboxyl*-^{14}C) methionine, but could be measured by the more sensitive colorimetric assay for peroxidase with *o*-dianisidine as electron donor. The amount found by this procedure in the supernatant solution after disruption and centrifugation of mycelium from 1 liter of culture was equivalent to 2.0 μg of horseradish peroxidase and was sufficient to account for the conversion of L-methionine to 3-methylthioacrylic acid by this route. Moreover, the enzyme, after sufficient concentration and purification, catalyzed the oxidative decarboxylation of L-(*carboxyl*-^{14}C) methionine and its cofactor requirements were similar to those of horseradish peroxidase. Samples concentrated and partially purified from the crude extract by ammonium sulfate precipitation and dialysis showed no activity in this assay even though adequate amounts, based on measurement of activity in the colorimetric assay, were present. Since they also suppressed the activity of horseradish peroxidase, and further purification gave preparations active in both assays, it is concluded that the cell homogenate contained an inhibitor. The presence of an inhibitor in crude preparations of horseradish peroxidase has been reported by MAZELIS.[5]

The peroxidase of *S. lincolnensis* was not extensively characterized, but showed a pH optimum with *o*-dianisidine as electron donor similar to that of other peroxidases. However, it does not exhibit typical heat stability and its molecular weight is higher than the value of 40,000~60,000 daltons common for plant peroxidases and the 90,000~150,000 of animal lacto- and myeloperoxidases.[15]

Although detection of peroxidase activity in *S. lincolnensis* supports the hypothesis that peroxidative decarboxylation of L-methionine is the initial step in the formation of 3-methylthioacrylic acid, lack of radioisotopic labelling from 3-(*methyl*-^{14}C) methylthiopropionamide suggests that this evidence should be treated with reserve. Absence of labelling by the intermediate may have been due to a permeability barrier preventing uptake, and it is possible that intermediates are bound to an enzyme complex and do not equilibrate with free compound in the cytoplasm, but the result may also mean that 3-methylthiopropionic and 3-methylthioacrylic acids in this culture of *Streptomyces* are formed *via* the keto acid, as proposed by ARIMA and his colleagues,[2] and not *via* the amide. The peroxidative route, if it exists, may be restricted to those organisms that accumulate the amides.

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