Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2

KAZUHIRO NAGAHAMA,¹ TAKAHIRA OGAWA,¹ TAKAO FUJII,¹ MASATO TAZAKI,² SUMIO TANASE,³ YOSHIMASA MORINO³ and HIDEO FUKUDA^{1*}

Departments of ¹Applied Microbial Technology and ²Industrial Chemistry, The Kumamoto Institute of Technology, Ikeda 4-22-1, Kumamoto 860, Japan

³Kumamoto University Medical School, Honjo 2-2-1, Kumamoto 860, Japan

(Received 2 May 1991; revised 26 June 1991; accepted 8 July 1991)

A novel ethylene-forming enzyme that catalyses the formation of ethylene from 2-oxoglutarate was purified from a cell-free extract of *Pseudomonas syringae* pv. *phaseolicola* PK2. It was purified about 2800-fold with an overall yield of 53% to a single band of protein after SDS-PAGE. The purified enzyme had a specific activity of 660 nmol ethylene min⁻¹ (mg protein)⁻¹. The molecular mass of the enzyme was approximately 36 kDa by gel filtration and 42 kDa by SDS-PAGE. The isoelectric point and optimum pH were 5.9 and ca. 7.0–7.5, respectively. There was no homology between the N-terminal amino acid sequence of the ethylene-forming enzyme of *Ps. syringae* pv. *phaseolicola* PK2 and the sequence of the ethylene-forming enzyme of the fungus *Penicillium digitatum* IFO 9372. However, the two enzymes have the following properties in common. The presence of 2-oxoglutarate, L-arginine, Fe²⁺ and oxygen is essential for the enzymic reaction. The enzymes are highly specific for 2-oxoglutarate as substrate and L-arginine as cofactor. EDTA, Tiron, DTNB [5,5'-dithio-bis(2-nitrobenzoate)] and hydrogen peroxide are all effective inhibitors.

Introduction

It is well known that ethylene is produced by various plants and can act as a plant hormone. There have also been many reports of ethylene production by aerobic heterotrophic micro-organisms. The ethylene-forming enzyme in plants, which converts 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, has not yet been isolated (Kende, 1989), but the characteristics of microbial ethylene-forming enzymes have gradually become clearer during the past decade. Two precursors of microbial ethylene production have been identified, namely 2-oxo-4-methylthiobutyric acid (KMBA), a deaminated derivative of L-methionine, and 2-oxoglutarate. The former precursor is used in so-called L-methionine-dependent ethylene-forming microbes and the latter in so-called 2-oxoglutarate-dependent ethylene-forming microbes.

Ince & Knowles (1986) reported the formation of

ethylene from KMBA by cell-free extracts of *Escherichia coli*. KMBA was converted to ethylene, methanethiol and probably carbon dioxide by a soluble enzyme system that required the presence of oxygen. Subsequently, Fukuda *et al.* (1989*b*) reported the purification of an ethylene-forming enzyme from the yeast *Cryptococcus albidus* that was active using the same *in vitro* system, and Ogawa *et al.* (1990) reported the mechanism of ethylene formation by this enzyme system. The physiology and biochemistry of ethylene production by *E. coli* SPAO were studied by Shipston & Bunch (1989), and Mansouri & Bunch (1989) examined the ethylenogenic capabilities of other bacteria grown in media supplemented with L-methionine and KMBA.

Fukuda *et al.* (1986) reported the partial purification of an ethylene-forming enzyme from the fungus *Penicillium digitatum* IFO 9372, which produces ethylene from 2-oxoglutarate. The enzyme system *in vitro* required L-arginine and Fe²⁺ in the presence of reducing agents such as DTT and oxygen as essential factors. This ethylene-forming enzyme has been purified to an electrophoretically homogeneous state (Fukuda *et al.*, 1989*a*). *Pseudomonas syringae* pv. *phaseolicola* PK2

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; KMBA, 2-oxo-4-methylthiobutyric acid; DTNB, 5,5'-dithio-bis(2-nitrobenzoate).

(Kudzu strain) is a 2-oxoglutarate-dependent ethylene-producing bacterium (Goto *et al.*, 1985; Goto & Hyodo, 1987). Recently, we constructed an ethylene-forming system using an extract of this bacterium (Nagahama *et al.*, 1991). This system was very similar to that prepared from *P. digitatum*, except for the presence of L-histidine. Therefore, it seems to be of interest to compare the properties of the ethylene-forming enzymes from *P. digitatum* and *Ps. syringae*.

In the present paper, we describe a method for the purification of the ethylene-forming enzyme from *Ps. syringae* pv. *phaseolicola* PK2 and show that the purified ethylene-forming enzyme has remarkably similar properties to those of the enzyme from *P. digitatum*.

Methods

Micro-organism and culture conditions. All experiments were done with *Pseudomonas syringae* pv. *phaseolicola* strain PK2, obtained from Professor M. Goto (Shizuoka University, Shizuoka, Japan). Methods for cultivation were those described previously (Nagahama *et al.*, 1991).

Purification of the ethylene-forming enzyme of Ps. syringae.

All steps of the purification were done at 4 °C unless otherwise indicated.

(1) **Preparation of a cell-free extract.** Cells were harvested from 9 litres of culture broth by centrifugation at 14000 *g* for 10 min. They were washed twice with deionized water and suspended in 250 ml potassium phosphate buffer (pH 7.0). The cells were disrupted by sonication (Branson Sonifier, model 350) for 5 min with 30 s intervals for cooling between each 30 s period of sonication. The intact cells and cell debris were removed by centrifugation (24000 *g*, 30 min) and the supernatant obtained was used as the cell-free extract.

(2) **Treatment with streptomycin sulphate.** To precipitate nucleic acids, streptomycin sulphate (at a final concentration of 1%, w/v) was added to the cell-free extract. After stirring the mixtures for 60 min, the sediment was removed by centrifugation at 24000 *g* for 20 min. The supernatant obtained was used as the crude enzyme.

(3) **Chromatography on Butyl-Toyoparl HW 650M.** Ammonium sulphate was added to the solution of crude enzyme to 30% saturation. The precipitate was removed by centrifugation at 24000 *g* for 20 min, and the supernatant was applied to a column (4 × 8 cm) of Butyl-Toyoparl HW 650M (Tosoh), which had been equilibrated with buffer A (10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-DTT) supplemented with ammonium sulphate to 30% saturation. The column was washed with the following buffers in turn: 100 ml of buffer A supplemented with ammonium sulphate to 30% saturation and 300 ml of buffer A supplemented with ammonium sulphate to 15% saturation. The flow rate was 2.05 ml cm⁻² h⁻¹ and the ethylene-forming enzyme was eluted from the column with 250 ml of buffer A. Active fractions were pooled and concentrated by ultrafiltration on a Diaflo YM10 membrane (Amicon). The concentrated solution of enzyme was dialysed in buffer B (10 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-DTT).

(4) **Chromatography on DEAE-Sepharose CL-6B.** The dialysed solution of enzyme was applied to a column (4 × 7 cm) of DEAE-Sepharose CL-6B (Pharmacia) which had been equilibrated with buffer B. The column was washed with about 1 vol. (80 ml) of buffer B and eluted with a linear gradient (800 ml) from 0 to 0.25 M-KCl in buffer B at a flow rate of 6 ml cm⁻² h⁻¹. Fractions containing ethylene-forming activity were pooled and concentrated by ultrafiltration on a Diaflo YM10 membrane.

(5) **Chromatography on Bio-Gel HT.** The concentrated solution of enzyme was dialysed against buffer C (20 mM-HEPES/NaOH buffer, pH 7.5, containing 1 mM-DTT) containing 1 mM-EDTA for 12 h and applied to a column (2.8 × 5 cm) of Bio-Gel HT (Bio-Rad), which had been pre-equilibrated with buffer C. For elution a linear gradient (300 ml) from buffer C to buffer B at a flow rate of 4.3 ml cm⁻² h⁻¹ was used. Active fractions were pooled and concentrated by ultrafiltration on a Diaflo YM10 membrane.

(6) **Gel filtration.** The concentrated solution of enzyme (2 ml) was applied to a column (2 × 88 cm) of Sephadex G-100 (Pharmacia) which had been pre-equilibrated with 10 mM-potassium phosphate buffer, pH 7.0 and eluted at a rate of 2.3 ml cm⁻² h⁻¹.

Purified ethylene-forming enzyme of *P. digitatum* IFO 9372. This was obtained from the cell-free extract as reported previously (Fukuda *et al.*, 1989a).

Amino-terminal sequence of the purified ethylene-forming enzymes of *Ps. syringae* and *P. digitatum*. The N-terminal amino acid residues of the purified enzymes of *Ps. syringae* and *P. digitatum* (20 µg, equivalent to approx. 480 pmol each) were determined by automated Edman sequencing with a 477A amino acid sequencer (Applied Biosystems).

Electrophoresis. SDS-PAGE was used to check the purity of the protein and to determine the molecular mass of the purified enzyme of *Ps. syringae* under denaturing conditions. SDS-PAGE was done in 12.5% (w/v) polyacrylamide slab gels by the method of Laemmli (1970). Isoelectric points (pIs) of the ethylene-forming enzymes of *Ps. syringae* and *P. digitatum* were determined with a PhastSystem and Pharmalyte 3-10 (Pharmacia).

Protein determinations. The concentration of protein was measured as the absorbance at 280 nm or by the Lowry method with bovine serum albumin as standard.

Ethylene formation. The assay for the formation of ethylene by *Ps. syringae* enzyme was done using a standard reaction mixture (1 ml) which contained 0.2 ml 200 mM-HEPES/NaOH buffer (pH 7.5), 0.1 ml 2.5 mM-2-oxoglutarate, 0.1 ml 2 mM-FeSO₄, 0.1 ml 20 mM-DTT, 0.1 ml 100 mM-L-histidine, 0.1 ml 2 mM-L-arginine, 0.2 ml of deionized water and 0.1 ml of a solution of enzyme. In the case of *P. digitatum*, the reaction mixture (1 ml) comprised 0.1 ml 200 mM-HEPES/NaOH buffer (pH 8.0), 0.1 ml 10 mM-2-oxoglutarate, 0.1 ml 0.75 mM-FeSO₄, 0.1 ml 20 mM-DTT, 0.1 ml 10 mM-L-arginine, 0.4 ml of deionized water and 0.1 ml of a solution of enzyme. A test-tube (15 mm diam., 13 ml capacity) containing each reaction mixture was sealed with a rubber stopper and then incubated on a reciprocal shaker (120 r.p.m.) at 25 °C for 10 min. After incubation, a sample of gas (1 ml) was withdrawn through the stopper with a gas syringe and the amount of ethylene in the sample was determined with a gas chromatograph (G3800; Yanako) under the following conditions: column size, 3 mm × 2 m; solid phase, active alumina; temperature, 100 °C; carrier gas, nitrogen at a flow rate of 40 ml min⁻¹; and detector, flame ionization. The rate of ethylene production was calculated as previously described (Fukuda *et al.*, 1984). One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 nmol ethylene min⁻¹ under the specified reaction conditions. The specific activity is expressed as units (mg protein)⁻¹.

Table 1. Purification of the ethylene-forming enzyme

The volume of culture broth used was 9 litres.

Purification step	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Yield (%)	Purification (-fold)
Cell-free extract	20000	4700	0.24	100	1
Streptomycin-treated fraction	7200	4800	0.67	102	3
Butyl-Toyopearl fraction	290	3500	12	74	50
DEAE-Sepharose fraction	18	2600	140	55	580
Bio-Gel fraction	9.4	4300	460	91	1900
Sephadex fraction	3.8	2500	660	53	2800

Table 2. Comparison of some properties of the ethylene-forming enzymes from *Ps. syringae* and *P. digitatum*

		<i>Ps. syringae</i>	<i>P. digitatum</i>
Molecular mass	Sephadex G-100	ca. 36 kDa	ca. 42 kDa
	SDS-PAGE	ca. 42 kDa	ca. 42 kDa
pI		5.9	5.9
pH	Optimum	ca. 7.0–7.5	ca. 7.0–7.5
	Stability (14 h at 5 °C)	ca. 6.0–8.0	ca. 6.0–8.0
Temperature	Optimum (for 10 min)	ca. 20–25 °C	25 °C
	Stability (for 10 min)	Stable below 30 °C	Stable below 30 °C
K_m value	Fe ²⁺	5.9×10^{-5} M	4.0×10^{-5} M
	L-Arginine	1.8×10^{-5} M	6.0×10^{-5} M
	2-Oxoglutarate	1.9×10^{-5} M	3.8×10^{-5} M

Results and Discussion

Purification of the ethylene-forming enzyme from *Ps. syringae* pv. *phaseolicola* PK2

The ethylene-forming enzyme from *Ps. syringae* pv. *phaseolicola* PK2 was purified from 9 litres of culture broth by column chromatography on Butyl-Toyopearl HW 650M, DEAE-Sepharose CL-6B, Bio-Gel HT and Sephadex G-100 as described in Methods (see Table 1). The enzyme was purified about 2800-fold from the cell-free extract to a specific activity of 660 units (mg protein)⁻¹ with a recovery of 53%. Only one band was observed when the final preparation of enzyme was subjected to SDS-PAGE and the gel was stained with Coomassie blue (data not shown). It should be noted that the yield from the DEAE-Sepharose column to the Bio-Gel column rose to 165%. This result was dependent on the conditions of dialysis because the activity could be approximately doubled or halved during the dialysis. When we did not include 1 mM-EDTA in buffer C during the dialysis, the activity of the fraction from the Bio-Gel

column decreased to about 50% of that of the fraction from the DEAE-Sepharose column (data not shown). Though these phenomena were reproducible, no explanation for them is available as yet.

Some properties of the purified ethylene-forming enzyme

Table 2 shows a comparison of some properties of the purified ethylene-forming enzyme of *Ps. syringae* with those of the purified ethylene-forming enzyme of *P. digitatum*. The molecular mass of the enzyme of *Ps. syringae*, calculated from gel-filtration on a column of Sephadex G-100, was 36 kDa; a value of 42 kDa was obtained by SDS-PAGE (data not shown). These results indicate that the enzyme of *Ps. syringae* is a monomeric protein. The isoelectric point was 5.9 on the PhastSystem (Pharmacia). The optimum pH and temperature were ca. 7.0–7.5 and ca. 20–25 °C, respectively. The K_m values for Fe²⁺, L-arginine and 2-oxoglutarate were 5.9×10^{-5} , 1.8×10^{-5} and 1.9×10^{-5} M, respectively. The properties of the ethylene-forming enzyme of *P. digitatum* are almost the same as that of *Ps. syringae* (see Table 2).

N-Terminal amino acid sequence of the ethylene-forming enzymes of *Ps. syringae* and *P. digitatum*

The N-terminal amino acid sequence of the ethylene-forming enzyme of *Ps. syringae* was determined and compared with that of *P. digitatum* (see Fig. 1). There was no homology between the N-terminal amino acid sequences of the two enzymes, although they have similar properties (Table 2; see also Tables 3 and 4). It should be noted that about 25% of the entire preparation of purified enzyme from *Ps. syringae* lacked an N-terminal methionine residue. Waller (1963) reported that only 40% of the polypeptide chains retain an N-terminal methionine residue; instead, about 50% of chains have alanine, serine or threonine at their amino termini. According to Hirel *et al.* (1989), the excision of the N-terminal methionine is catalysed by a methionyl-amino

Ps. syringae pv. *phaseolicola* PK2

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Met-Thr-Asn-Leu-Gln-Thr-Phe-Glu-Leu-Pro-Thr-Glu-Val-Thr-Gly-

16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
X-Ala-Ala-Asp-Ile-Ser-Leu-Gly-Arg-Ala-Leu-Ile-Gln-Ala-Lys

P. digitatum

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Leu-Ala-Pro-Pro-Ala-Pro-Ser-Asn-Leu-Gly-Ser-Thr-Met-Pro-Pro-

16 17 18 19 20 21 22 23 24 25
Asn-Tyr-Val-Ala-X-Val-Gly-Gln-Leu-Lys

Fig. 1. Comparison of the N-terminal amino acid sequences of the ethylene-forming enzymes from *Ps. syringae* and *P. digitatum*.

peptidase. The ethylene-forming enzyme of *Ps. syringae* may therefore be susceptible to such a peptidase.

Substrate and cofactor specificity

The optimal concentrations of the compounds essential for the formation of ethylene by the purified ethylene-forming enzyme of *Ps. syringae* were determined. The optimal concentrations of 2-oxoglutarate, Fe^{2+} , L-histidine and L-arginine were ca. 0.2–0.4 mM, 0.2–0.4 mM, 2–10 mM and 0.1–0.2 mM, respectively (data not shown). It should be noted that (1) 2-oxoglutarate, Fe^{2+} and L-arginine were absolutely essential for the formation of ethylene, there being no activity when any of these

compounds was omitted from the reaction mixture; (2) the optimal concentrations of these three compounds were almost the same (ca. 0.2 mM); (3) L-histidine was not essential for the formation of ethylene; and (4) DTT was not needed at all. It is noteworthy that the enzyme system required oxygen as an essential factor (data not shown). The substrate specificity and cofactor specificity of the ethylene-forming enzyme of *Ps. syringae* were examined by replacing 2-oxoglutarate, L-arginine and L-histidine, respectively, with various alternative compounds. The ethylene-forming enzyme was highly specific for 2-oxoglutarate (data not shown) and L-arginine (see Table 3*a*) and L-histidine (see Table 3*b*). The respective ethylene-forming activities in the pres-

Table 3. *Cofactor specificity of the ethylene-forming enzyme from Ps. syringae*

The specific activity of the ethylene-forming enzyme used was 660 units (mg protein)⁻¹.

(a)		(b)	
Cofactor (0.2 mM)	Relative ethylene-forming	Cofactor (10 mM)	Relative ethylene-forming
L-Arginine	100	Imidazole	62
D-Arginine	3	Imidazole-4-acetic acid	37
L-Canavanine sulphate	7	N-Acetylimidazole	57
L-Citrulline	0	L-Histidine	100
L-Ornithine.HCl	0	D-Histidine	89
L-Arginyl-L-arginine	0	1-Methyl-L-histidine	59
L-Arginyl-L-glutamic acid	0	Histamine	45
Poly-L-arginine	0	N-Acetylhistamine	50
N ⁹ -Nitro-L-arginine	0	L-Carnosine	11
N ⁹ -Tosyl-L-arginine	0	L-Histidyl-L-glycine	33
N-Carbobenzoxy-L-arginine	0	None	50
None	0		

Table 4. *Effects of various reagents on the formation of ethylene*

All concentrations were 1 mM, except for superoxide dismutase [150 units (ml reaction mixture)⁻¹], hydroquinone (10 mM), catalase [0.05 mg (ml reaction mixture)⁻¹] and H₂O₂ (0.03%). The specific activity of the ethylene-forming enzyme used was 660 units (mg protein)

Reagent	Relative ethylene-forming activity (%)
Control	100
Respiratory inhibitor NaN ₃	90
Free radical reagents	
Superoxide dismutase	120
Mannitol	90
Sodium benzoate	100
n-Propyl gallate	1
Uric acid	90
Hydroquinone	60
Transition metals and SH-reagents	
CoCl ₂	20
CuSO ₄	50
MnCl ₂	6
DTNB	0.7
Chelating reagents	
EDTA(Na)	1
Tiron*	0.8
Others	
Catalase	200
H ₂ O ₂	0.7
Ascorbate	130

* 4,5-dihydroxy-1,3-benzene disulphonic acid.

ence of D-arginine and L-canavanine sulphate were only 3% and 7% of that in the presence of L-arginine. From Table 3 it can be seen that (1) the activity with D-histidine was about 90% of that with L-histidine; (2) the activity without L-histidine was about 50% of that in the presence of L-histidine; and (3) imidazole was somewhat stimulatory. These results strongly suggest that the role of L-histidine is not that of a ligand, as suggested in a previous report (see Fig. 4 of Nagahama *et al.*, 1991), but that of a scavenger of active species of oxygen (Levine, 1983).

Inhibitors and activators of the ethylene-forming enzyme

The effects of a number of potential inhibitors and other reagents on the ethylene-forming activity of *Ps. syringae* were examined (Table 4). Strong inhibition by the chelating reagents EDTA and Tiron was observed, suggesting that some kind of complex with Fe(II) is involved in the enzymic reaction, as shown in Fig. 4 of our previous report (Nagahama *et al.*, 1991). Divalent transition metals, such as Co(II), Cu(II) and Mn(II) may compete with Fe(II) in the formation of such a complex

and interfere with the ethylene-forming activity of the enzyme. Some SH-groups in the enzyme may be important for activity given the inhibitory effect of DTNB. Superoxide and hydrogen peroxide are not involved in the reaction since superoxide dismutase and catalase were not inhibitory. The activation of the reaction by catalase implies the formation of hydrogen peroxide during the reaction. The involvement of hydroxyl radicals is also unlikely since the effects of mannitol and sodium benzoate were small. These results imply co-ordination with Fe²⁺ which may be loosely bound to the enzyme through its SH-groups. Other scavengers of free radicals, namely propyl gallate and hydroquinone, inhibited the reaction to a considerable extent. This effect should not, however, be attributed directly to the radical-scavenging properties of these reagents. They may also form complexes with Fe(II) and inhibit the reaction in a similar manner to EDTA. These results show that the purified ethylene-forming enzyme of *Ps. syringae* has remarkably similar properties to the enzyme from *P. digitatum* (Fukuda *et al.*, 1989a). We are now studying the mechanism of ethylene formation by the ethylene-forming enzymes from *Ps. syringae* and *P. digitatum*.

References

- FUKUDA, H., FUJII, T. & OGAWA, T. (1984). Microbial production of C₂-hydrocarbons, ethane, ethylene and acetylene. *Agricultural and Biological Chemistry* **48**, 1363–1365.
- FUKUDA, H., FUJII, T. & OGAWA, T. (1986). Preparation of a cell-free ethylene-forming system from *Penicillium digitatum*. *Agricultural and Biological Chemistry* **50**, 977–981.
- FUKUDA, H., KITAJIMA, H., FUJII, T., TAZAKI, M. & OGAWA, T. (1989a). Purification and some properties of a novel ethylene-forming enzyme produced by *Penicillium digitatum*. *FEMS Microbiology Letters* **59**, 1–6.
- FUKUDA, H., TAKAHASHI, M., FUJII, T., TAZAKI, M. & OGAWA, T. (1989b). An NADH:Fe(III)EDTA oxidoreductase from *Cryptococcus albidus*: an enzyme involved in ethylene production *in vivo*? *FEMS Microbiology Letters* **60**, 107–112.
- GOTO, M. & HYODO, H. (1987). Ethylene production by cell-free extract of the Kudzu strain of *Pseudomonas syringae* pv. *phaseolicola*. *Plant and Cell Physiology* **28**, 405–414.
- GOTO, M., ISHIDA, Y., TAKIKAWA, Y. & HYODO, H. (1985). Ethylene production by the Kudzu strains of *Pseudomonas syringae* pv. *phaseolicola* causing halo blight in *Pueraria lobata* (Willd) Ohwi. *Plant and Cell Physiology* **26**, 141–150.
- HIREL, PH.-H., SCHMITTER, J. M., DESSEN, P., FAYAT, G. & BLANQUEST, S. (1989). Extent of N-terminal methionine excision from *Escherichia coli* protein is governed by the side-chain length of the penultimate amino acid. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 8247–8251.
- INCE, J. E. & KNOWLES, C. J. (1986). Ethylene formation by cell-free extract of *Escherichia coli*. *Archives of Microbiology* **146**, 151–158.
- KENDE, H. (1989). Enzymes of ethylene biosynthesis. *Plant Physiology* **91**, 1–4.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–686.

- LEVINE, R. L. (1983). Oxidative modification of glutamine synthetase. *Journal of Biological Chemistry* **258**, 11828–11833.
- MANSOURI, S. & BUNCH, A. W. (1989). Bacterial ethylene synthesis from 2-oxo-4-thiobutyric acid and from methionine. *Journal of General Microbiology* **135**, 2819–2827.
- NAGAHAMA, K., OGAWA, T., FUJII, T., TAZAKI, M., GOTO, M. & FUKUDA, H. (1991). L-Arginine is essential for the formation *in vitro* of ethylene by an extract of *Pseudomonas syringae*. *Journal of General Microbiology* **137**, 1641–1646.
- OGAWA, T., TAKAHASHI, M., FUJII, T., TAZAKI, M. & FUKUDA, H. (1990). The role of NADH:EDTA(III) oxidoreductase in ethylene formation from 2-keto-4-methylthiobutyrate. *Journal of Fermentation and Bioengineering* **69**, 287–291.
- SHIPSTON, N. & BUNCH, A. W. (1989). The physiology of L-methionine catabolism to the secondary metabolite ethylene by *Escherichia coli*. *Journal of General Microbiology* **135**, 1489–1497.
- WALLER, J. P. (1963). The NH₂-terminal residues of the proteins from cell-free extracts of *Escherichia coli*. *Journal of Molecular Biology* **7**, 483–496.