L-Arginine is essential for the formation *in vitro* of ethylene by an extract of *Pseudomonas syringae*

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(Received 5 November 1990; revised 25 January 1991; accepted 25 March 1991)

A system was developed for the formation of ethylene *in vitro* by an extract of *Pseudomonas syringae* pv. *phaseolicola* PK2. The ethylene-forming activity of a cell-free extract of this bacterium measured in a system reported previously was almost completely lost when the cell-free extract was dialysed against potassium phosphate buffer for 24 h at 4 °C. When the fraction of cell-free extract with a molecular mass < 10 kDa (SupI) was added back to the enzyme fraction after gel filtration of the cell-free extract, the enzymic activity increased to about four times that of the gel-filtered crude enzyme. The action of SupI could be reproduced by the addition of L-arginine. The complete system for the formation of ethylene under aerobic conditions *in vitro* required 0.25 mM-2-oxoglutarate, 0.2 mM-FeSO₄, 2 mM-DTT, 10 mM-L-histidine and 0.2 mM-L-arginine. The cofactor specificity was examined by replacing L-arginine or L-histidine with various analogues, but none of them were effective. The components of this system, with the exception of L-histidine, were similar to those of a system derived from the ethylene-producing, plant pathogenic fungus *Penicillium digitatum* which also produced ethylene *in vitro* in a reaction dependent on 2-oxoglutarate. The intermediates in the ethylene-forming reaction are postulated and the roles of L-arginine and L-histidine in the formation of ethylene by *Ps. syringae* are discussed.

Introduction

There are two biosynthetic pathways that lead to ethylene production in micro-organisms. One pathway involves 2-oxoglutarate (Fukuda *et al.*, 1986, 1989a) and the other 2-keto-4-methylthiobutyric acid (KMBA) (Fukuda *et al.*, 1989b, c; Ogawa *et al.*, 1990). We refer to organisms that use the former pathway as 2-oxoglutarate-dependent ethylene-producing micro-organisms, e.g. *Penicillium digitatum*, and to organisms that use the latter pathway as methionine-dependent ethylene-producing micro-organisms, e.g. *Cryptococcus albidus*. Ethylene-forming enzymes have been purified to electrophoretic homogeneity from both *P. digitatum* (Fukuda *et al.*, 1989a) and *C. albidus* (Fukuda *et al.*, 1989c).

A number of ethylene-producing strains of bacteria have been reported and after a series of studies with *Escherichia coli* B SPAO, Primrose (1976, 1977) proposed that KMBA was an intermediate in ethylene biosynthesis. Ince & Knowles (1986) showed that ethylene was

synthesized by a cell-free extract of *E. coli* B SPAO incubated with KMBA, NAD(P)H, Fe(III) chelated to EDTA and oxygen, and discussed the possibility that all bacterial ethylene production might occur via the route demonstrated for *E. coli*. The physiology and biochemistry of ethylene production by *E. coli* and by other selected bacteria, including a strain of *Pseudomonas aeruginosa*, has since been investigated further (Shipston & Bunch, 1989; Mansouri & Bunch, 1989).

The bacterium *Pseudomonas syringae* pv. *phaseolicola* PK2 (Kudzu strain), which in Japan causes halo blight of the viny weed *Pueraria lobata* (Willd) Ohwi (common name Kudzu), is known to produce ethylene (Goto *et al.*, 1985). Goto & Hyodo (1987) reported that ethylene was produced when a cell-free extract of this bacterium was incubated under aerobic conditions with 0.5 mM-2-oxoglutarate, 0.5 mM-FeSO₄, 10 mM-L-histidine and 5 mM-DTT in 50 mM-HEPES/NaOH (pH 7.0). However, the ethylene-forming activity of this system was completely lost when the cell-free extract was dialysed against 10 mM-potassium phosphate buffer (pH 7.0) for 24 h at 4 °C, indicating the loss, during dialysis, of a factor essential for enzymic activity.

Abbreviation: KMBA, 2-keto-4-methylthiobutyric acid.

The purpose of this study was to identify the factor(s) essential for the ethylene-forming activity of *Ps. syringae* pv. *phaseolicola* and to construct an ethylene-forming system *in vitro*.

Methods

Micro-organism and culture conditions. All experiments were done with *Pseudomonas syringae* pv. *phaseolicola* strain PK2. The strain was stored at 4 °C on NB medium containing 0.5% (w/v) glucose and 2% (w/v) agar. NB medium comprised 0.5% Polypepton, 0.3% meat extract, 0.2% yeast extract and 0.2% NaCl (pH 7.0). A loopful of bacteria was inoculated into a 100 ml Erlenmeyer flask containing 20 ml of NB medium supplemented with 0.5% glucose and the resulting seed culture was grown up at 30 °C on a rotary shaker, at 180 r.p.m., for 24 h. Then 4 ml of the seed culture was transferred to a 500 ml Erlenmeyer flask containing 200 ml 0.5% glucose in NB medium and placed on rotary shaker for 10–12 h under the same conditions as for the seed culture.

Preparation of cell-free extract. Cells were harvested from 900 ml of culture broth by centrifugation at 14000 g for 10 min. They were washed twice with deionized water and suspended in 90 ml 10 mM-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. Intact cells and cell debris were removed by centrifugation (24000 g, at 4 °C for 30 min) and the supernatant obtained was used as the cell-free extract.

Preparation of partially purified enzyme and a low molecular mass fraction. The cell-free extract was concentrated by ultrafiltration on a DIAF YM 10 membrane (Amicon) to give two fractions, a crude enzyme fraction containing macromolecules with molecular masses >10 kDa and a fraction (SupI) containing substances with molecular masses <10 kDa. The SupI fraction was concentrated by lyophilization and the optical density at 280 nm was adjusted by adding deionized water. The crude enzyme solution was applied to a gel-filtration column (2.6 × 80 cm, gel volume 425 ml) of Sephadex G-75 (Pharmacia) equilibrated with 50 mM-potassium phosphate buffer (pH 7.0). Eluted fractions that had ethylene-forming activity were pooled. This pool was termed Fraction I and was used as partially purified enzyme.

Ethylene formation. This was determined using a modified version of the reaction mixture of Fukuda *et al.* (1986) and of Goto & Hyodo (1987), containing: 0.2 ml 200 mM-HEPES/NaOH buffer (pH 8.0), 0.1 ml 5 mM-2-oxoglutarate, 0.1 ml 0.75 mM-FeSO₄, 0.1 ml 20 mM-DTT, 0.1 ml 100 mM-L-histidine, 0.2 ml of deionized water and 0.1 ml of enzyme solution. A test-tube (15 mm diameter, 13 ml capacity) containing the reaction mixture (1 ml) was sealed with a rubber stopper and then incubated on a reciprocal shaker (120 r.p.m.) at 25 °C for 10 min. In the case of ethylene production by living cells, culture broth (1 ml) was transferred to a sterile test-tube (18 mm diameter, 34 ml capacity). The test-tube was sealed with a rubber stopper and then incubated on a reciprocal shaker (120 r.p.m.) at 30 °C for 1 h. 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 (flow rate 40 ml min⁻¹); detection, flame ionization. The rate of ethylene formation was calculated as previously described (Fukuda *et al.*, 1984).

Amount of cells. This was determined from the optical density at 610 nm or from the dry weight.

Glucose concentration. The concentration of glucose in supernatants was measured by the Glucose B-Test (Wako Pure Chemicals).

Protein concentration. This was measured as the absorbance at 280 nm or by the Lowry method with BSA as the standard.

Results

Effect of SupI addition on ethylene-forming activity

The ethylene-forming activities of cell-free extract, treated by dialysis for 24 h at 4 °C, the crude enzyme fraction and the SupI fraction (obtained by ultrafiltration; see Methods), were measured (Table 1). The modified *in vitro* system of Fukuda *et al.* (1986) and Goto & Hyodo (1987) was used for measurements of ethylene-forming activity. Activities recovered, as a percentage of total activity, of the crude enzyme and cell-free extract after dialysis were 49% and 3% of the total activity in the cell-free extract, respectively. However, the activities recovered after addition of SupI to the crude enzyme fraction and the cell-free extract after dialysis increased to 87% and 25% of the total activity in the cell-free extract. Reactivation of the system may be more complex in the case of dialysed extract, because of inactivation of the enzyme occurred during dialysis, and the activity of dialysed extract was very low. Anyhow, this result indicates the presence of a factor in SupI that is essential for ethylene-forming activity. This factor was stable to heating at 121 °C for 5 min (see Table 1). Therefore, the essential component is heat-stable and has a molecular mass <10 kDa.

Table 1. *Effects on ethylene-forming activity of the addition of SupI to various fractions*

The volume of enzyme solution used was 10 ml in all cases. The protein concentration of cell-free extract was 9.59 mg ml⁻¹; it was dialysed, where indicated, against 10 mM-potassium phosphate buffer (pH 7.0) for 24 h at 4 °C. Crude enzyme is the fraction of cell-free extract which was concentrated on a DIAF YM 10 ultrafiltration membrane; the solution was made up to 10 ml with 10 mM-potassium phosphate buffer, pH 7.0. SupI is the fraction with a molecular mass <10 kDa which passed through a DIAF YM 10 membrane; the protein concentration of added SupI was 1.08 mg ml⁻¹.

Enzyme source	SupI added	Ethylene-forming activity (nl ml ⁻¹ h ⁻¹)	Relative activity (%)
Cell-free extract	—	1522	100
Crude enzyme	—	748	49
Cell-free extract after dialysis	—	49	3
Crude enzyme	+	1319	87
Crude enzyme	+*	1368	90
Cell-free extract after dialysis	+	387	25

* Heat-treated (121 °C, 5 min) SupI was used.

Fig. 1. Elution from a column of Sephadex G-75 of crude enzyme from *Ps. syringae* pv. *phaseolicola* PK2. The fraction vol. was 4 ml. The protein content (○) of each fraction, together with the ethylene-forming activities of (△) partially purified enzyme, (▲) partially purified enzyme plus SupI and (■) fractions from 80–120 plus Fraction I are shown. Fraction I comprises fractions 40–59 that have activity in the ethylene-forming assay. The protein concentration of SupI used was 11.65 mg ml⁻¹.

Chromatography on Sephadex G-75 of crude enzyme

In order to obtain a partially purified enzyme without the SupI fraction, gel filtration on a column of Sephadex G-75 was performed using a solution of crude enzyme that had been concentrated about ten-fold by ultrafiltration (Fig. 1). The ethylene-forming activity of the enzyme was very low and the activity recovered was about 21% of that of the crude enzyme. The total ethylene-forming activity after addition of SupI was about four times that without SupI. Fractions 40–59 were pooled as the ethylene-forming enzyme fraction (termed Fraction I). Fraction I was added to each fraction from 80–120, and from a comparison of the activities of Fraction I alone, and of the combined fraction, it was found that a compound with properties similar to those of SupI was present in fractions 80–100. To determine the essential factor in SupI, its properties were examined, and found to be as follows: ninhydrin-positive, dialysable and heat-stable. The essential factor was considered, therefore, to be a peptide or an amino acid.

Next we examined the effects of the addition of Polypepton, meat extract, yeast extract, NZ amine (Wako Pure Chemicals) and Casamino acids (vitamin-free) to Fraction I. Since all these additives were effective (data not shown), amino acids were added separately to the reaction mixture (Table 2). It was found that SupI could be essentially replaced by L-arginine.

Table 2. Effects of amino acids on the ethylene-forming activity of Fraction I

The reaction mixture contained 40 mM-HEPES/NaOH buffer (pH 8.0), 0.075 mM-FeSO₄, 2 mM-DTT, 10 mM-histidine and 0.5 mM-2-oxoglutarate. The concentration of Fraction I protein in each reaction mixture was 0.99 mg ml⁻¹. The concentrations of amino acids and Casamino acids were 1 mM and 1 mg per reaction mixture, respectively.

Amino-acid added	Ethylene-forming activity (nl ml ⁻¹ h ⁻¹)	Amino acid added	Ethylene-forming activity (nl ml ⁻¹ h ⁻¹)
Glycine	294	L-Tryptophane	251
L-Alanine	271	L-Tyrosine	228
L-Valine	251	L-Asparagine	261
L-Leucine	280	L-Glutamine	341
L-Isoleucine	278	L-Aspartic acid	256
L-Phenylalanine	246	L-Glutamic acid	253
L-Proline	249	L-Lysine	194
L-Serine	278	L-Arginine	3079
L-Threonine	266	L-Histidine	239
L-Cysteine	109	Casamino acids	1580
L-Methionine	249	None	298

Reconstruction of an ethylene-forming system *in vitro*

The optimal concentrations of the compounds required for the formation of ethylene by the partially purified enzyme were determined (Fig. 2). The optimal concentrations of 2-oxoglutarate, FeSO₄, DTT, L-histidine and L-arginine were 0.25, 0.2, 2, 10 and 0.2 mM, respectively. We were able to reconstruct an ethylene-forming system *in vitro* using the partially purified enzyme from *Ps. syringae* pv. *phaseolicola* PK2. The standard reaction mixture, incubated under aerobic conditions, contained the following: 0.2 ml 200 mM-HEPES/NaOH buffer (pH 8.0), 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 enzyme solution (total vol. 1.0 ml).

In order to ascertain the necessity for ethylene formation of each component of the standard reaction mixture, each compound separately was omitted from the mixture (Table 3). The presence of 2-oxoglutarate, Fe²⁺ and L-arginine was essential and DTT and L-histidine markedly stimulated the reaction.

Effects of the addition of analogues of L-arginine and L-histidine on ethylene formation

The cofactor specificity of the ethylene-forming enzyme was examined by replacing L-arginine or L-histidine with various compounds (Table 4). Ethylene-forming activity in the presence of D-arginine and L-canavanine was 11%

Fig. 2. Effects of varying the concentrations of essential components in the reaction system. When the concentration of one component was changed, those of the others were fixed as follows: 0.25 mM-2-oxoglutarate, 2 mM-DTT, 0.2 mM-FeSO₄, 10 mM-L-histidine and 0.2 mM-L-arginine. The concentration of Fraction I protein added to each reaction mixture was 0.99 mg ml⁻¹.

Table 3. Effects of omission of each component from the standard reaction mixture on the ethylene-forming activity of Fraction I

The components of each reaction mixture were the same as those in the standard reaction mixture except for the omission of the cited component. The concentration of Fraction I protein in each reaction mixture was 0.99 mg ml⁻¹.

Component omitted	Ethylene-forming activity (nl ml ⁻¹ h ⁻¹)	Relative activity (%)
None	4637	100
2-Oxoglutarate	0	0
FeSO ₄	201	4
DTT	1852	40
L-Histidine	1114	24
L-Arginine	208	4
Fraction I	0	0

and 12%, respectively, of that in the presence of L-arginine. None of the L-histidine analogues tested could replace L-histidine, although imidazole enhanced ethylene-forming activity to a small extent.

Comparison of the time-course of ethylene formation in the cell-free system with that in living cells

The time-courses of ethylene formation both in the cell-free system (*in vitro*) and in living cells (*in vivo*) are shown in Fig. 3. The two time-courses were somewhat dissimilar, but the two peaks of the rate of ethylene

Table 4. Effects of the addition of analogues of L-arginine or L-histidine on the ethylene-forming activity of Fraction I

The concentrations of components in each reaction mixture, except for L-arginine or L-histidine, were the same as in the standard reaction mixture. The concentration of Fraction I protein in each reaction mixture was 0.99 mg ml⁻¹.

Compound added	Final concn (mM)	Ethylene-forming activity (nl ml ⁻¹ h ⁻¹)	Relative activity (%)
L-Arginine	0.2	5618	100
D-Arginine	0.2	596	11
L-Canavanine	0.2	680	12
Poly-L-arginine	0.2	473	8
None		375	7
L-Histidine	10.0	5021	100
	1.0	2961	59
Histamine	10.0	1602	32
	1.0	1351	27
Imidazole	10.0	1985	40
	1.0	1284	26
None		1311	26

formation almost coincided. We therefore believe the equivalent of this cell-free ethylene-forming system operates in living cells.

Discussion

In this work, we have described the construction of an ethylene-forming system *in vitro* using cell-free extracts of *Ps. syringae* pv. *phaseolicola* PK2. Although Goto &

Fig. 3. Time-courses of ethylene formation by living cells and by the cell-free system. ●, Cell concentration (OD_{610}); ▲, glucose concentration; ○, pH; □, ethylene-forming activity of living cells; ■, ethylene-forming activity of the cell-free system.

Hyodo (1987) constructed an ethylene-forming system *in vitro* from this bacterium, the activity of the cell-free extract used was completely lost as a result of dialysis. This indicated that dialysable, low-molecular-mass substances present in cell-free extracts were essential factors for the ethylene formation. We tried to separate these low-molecular-mass substances from the cell-free extract, and we confirmed the existence of a factor with a molecular mass < 10 kDa that was essential for ethylene formation. This factor could essentially be replaced by L-arginine (see Table 2). L-Arginine is also required as an essential component in an ethylene-forming system derived from *P. digitatum* (Fukuda *et al.*, 1986). The

ethylene-forming system derived from *Ps. syringae* pv. *phaseolicola* PK2 was similar to that from *P. digitatum*, with the exception of the stimulatory effect of L-histidine.

The requirement for L-arginine as well as for Fe(II) ions, 2-oxoglutarate and L-histidine is best understood, at this stage of our investigation, in terms of the hypothetical reaction mechanism described below, if we assume that the ethylene-forming enzymes from *Ps. syringae* pv. *phaseolicola* PK2 and *P. digitatum* act in a closely related manner. Under the optimum conditions (see Fig. 2), the concentrations of L-arginine, Fe(II) and 2-oxoglutarate are too low for these chemical species to interact with each other in bulk solution. This situation means, simply, that these species must be concentrated within certain domain(s) of the enzyme in some special way. Thus, the active site of the enzyme can be considered hypothetically as an Fe(II) complex bound to the enzyme through an appropriate ligand atom Y. The complex also binds L-arginine and 2-oxoglutarate as a Schiff-base structure to form an intermediate, as shown in Fig. 4(a).

The major role of L-histidine is most probably attributable to its imidazole ring, since imidazole itself accelerated the formation of ethylene to a small but significant extent. The ring may possibly coordinate with the intermediate Fe(II) complex, modifying the energy levels of 3d electrons of Fe(II) so that it becomes easier to bind molecular oxygen. Then the intermediate reacts with oxygen to form an unstable Fe(IV) complex of the peroxo-type (see Fig. 4b), the 2-oxoglutarate moiety of which is decomposed to ethylene and three molecules of carbon dioxide. During these oxidation processes, L-arginine, L-histidine and Fe(II) are regenerated and, thus they can be regarded as cofactors from a mechanistic point of view. The chemical behaviour of the enzyme isolated from *P. digitatum* does not exclude this hypothetical mechanism, except that no relevant information is available about the imidazole ring included in the

Fig. 4. Model of the proposed intermediate consisting of the ethylene-forming enzyme, 2-oxoglutarate, L-arginine and the imidazole compound. (a) The intermediate before reaction with oxygen and (b) after reaction. The carbon atoms from 2-oxoglutarate that are converted to ethylene are indicated (●).

scheme in Fig. 4. The differences between the two enzymes are of great interest to us. Studies are now in progress on the further purification of the ethylene-forming enzyme, using the *in vitro* system described in this report, and on the biochemical mechanism of the formation of ethylene by *Ps. syringae*.

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