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D-AMINO-ACID-STIMULATED ETHYLENE PRODUCTION: MOLECULAR REQUIREMENTS FOR THE STIMULATION AND A POSSIBLE RECEPTOR SITE

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Key Word Index—*Xanthium pennsylvanicum*; Compositae; stimulation of ethylene production; molecular requirements; kinetic analysis; receptor site; D-amino acid.

Abstract—Various kinds of D-amino acids enhanced ethylene production in the cotyledonary segments of *Xanthium pennsylvanicum* seeds. To be effective they required not only their D-configuration but also the presence of α-NH_2, α-COOH and a hydrophobic as well as a bulky side chain. Moreover, they exhibited almost the same dissociation constant in kinetic analysis. On the other hand, L-phenylalanine and D-serine, which themselves were ineffective in the stimulation of ethylene production, competitively inhibited D-phenylalanine-stimulated ethylene production. Thus, the D-amino-acid-stimulated ethylene production was explained by assuming the existence in cells of D-amino-acid receptor site associated with ethylene synthesis.

INTRODUCTION

D-Amino acids are not normal constituents of plants, but they have been isolated from several species in free form or as conjugates with other L-amino or organic acids, although their physiological functions, if any, are still obscure [1–4]. In the study of C_2H_4 formation in seed tissues, we found that D-amino acids stimulated C_2H_4 production in segments from the seeds of cocklebur, pumpkin, sunflower and mung bean, whereas the corresponding L-amino acids failed to elicit such an effect [5]. Nowadays, L-methionine is widely accepted as a general precursor of C_2H_4 in plants [6]. It seemed, however, unlikely that D-amino acids exerted their stimulatory effect on C_2H_4 production by acting as substrates for C_2H_4 production through their conversion to L-methionine [5]. The effectiveness in the stimulation was different among various D-amino acids. In this paper, we describe in more detail the structural requirements of D-amino acids for the stimulation of C_2H_4 production and discuss the mechanism of action of D-amino acids in terms of a D-amino-acid receptor site, which was deduced from the structural requirements as well as kinetic analysis.

RESULTS AND DISCUSSION

Molecular requirements for stimulation

As reported in the previous paper [5], D- but not L-isomers of phenylalanine and valine cause stimulation of C_2H_4 production in cotyledonary segments from cocklebur seeds. Hence, the molecular requirements of amino acids for the stimulation of C_2H_4 production were examined by testing the effectiveness of various analogues of phenylalanine and valine (Table 1). Both D-phenylalanine and D-valine stimulated C_2H_4 production *ca* 2.8-fold over the control, whereas their L-isomers were quite ineffective, as already mentioned [5]. Replacement with hydrogen of either the α-amino group (to form 3-phenylpropionic acid and isovaleric acid) or the α-carboxyl group (to form 2-phenylethylamine and isobutylamine) of each of these acids resulted in little or no

Table 1. Effects of analogues of D-phenylalanine and D-valine on C_2H_4 production in the cotyledonary segments of cocklebur seeds

Chemicals (10 mM)	Relative amount (RA) (mean ± s.e.)
Experiment 1	
H_2O (control)	1.00*
D-Phenylalanine	2.76 ± 0.14
L-Phenylalanine	1.07 ± 0.09
3-Phenylpropionic acid†	0.97 ± 0.03
2-Phenylethylamine†	1.25 ± 0.07
3-Phenylpyruvic acid†	1.07 ± 0.06
N-Acetyl-D-β-phenylalanine†	1.00 ± 0.17
N-Formyl-D-β-phenylalanine†	1.26 ± 0.17
Experiment 2	
H_2O (control)	1.00*
D-Valine	2.82 ± 0.10
L-Valine	0.87 ± 0.07
Isovaleric acid†	1.02 ± 0.09
Isobutylamine†	0.85 ± 0.04
Experiment 3	
H_2O (control)	1.00*
Glycine	1.05 ± 0.04

*Absolute values (nl/hr/segment): experiment 1, 0.029; experiment 2, 0.035; experiment 3, 0.027.

†Adjusted to pH 6.5–7.0 with HCl or NaOH.

Table 2. Effects of D-amino acids with different side chain structure on C_2H_4 production of the cotyledonary segments of cocklebur seeds

D-Amino acid (10 mM)		Relative amount* (mean ± s.e.)
1. Length of side chain		
Glycine	$-H$	1.06 ± 0.08
Alanine	$-Me$	1.36 ± 0.11
α-Amino-*n*-butyric acid	$-CH_2-Me$	1.37 ± 0.06
Norvaline	$-CH_2-CH_2-Me$	1.41 ± 0.00
Norleucine	$-CH_2-CH_2-CH_2-Me$	1.44 ± 0.21
2. Branching in side chain		
Valine	$-CH(Me)_2$	2.25 ± 0.15
Leucine	$-CH_2-CH(Me)_2$	1.76 ± 0.23
Isoleucine	$-CH(Me)-CH_2-Me$	2.23 ± 0.16
Phenylalanine	$-CH_2Ph$	2.43 ± 0.03
3. —OH substitution		
Serine	$-CH_2-OH$	1.01 ± 0.04
Threonine	$-CH(OH)-Me$	1.77 ± 0.13
Phenylalanine†	$-CH_2Ph$	1.27 ± 0.05
Tyrosine†	$-CH_2-C_6H_4-p$-OH	1.09 ± 0.04
4. Charged groups in side chain		
Aspartate‡	$-CH_2-COO^-$	1.03 ± 0.11
Glutamate‡	$-CH_2-CH_2-COO^-$	1.11 ± 0.10
Ornithine‡	$-CH_2-CH_2-CH_2-\overset{+}{N}H_3$	0.96 ± 0.05
Arginine‡	$-(CH_2)_3-NH-C(NH_2)=\overset{+}{N}H_2$	1.05 ± 0.05
5. Sulphur in side chain		
Cysteine	$-CH_2-SH$	0.58 ± 0.07
Methionine	$-CH_2-CH_2-S-Me$	2.14 ± 0.10
Ethionine	$-CH_2-CH_2-S-CH_2-Me$	2.54 ± 0.10

*H_2O control, 0.033 nl/hr/segment.
†2 mM.
‡Adjusted to pH 5.5–6.5 with NaOH or HCl.

stimulation of C_2H_4 production. Acylation and formylation of the α-amino group of D-phenylalanine (to form *N*-acetyl-D-β-phenylalanine and *N*-formyl-D-β-phenylalanine) also inactivated the molecule with respect to C_2H_4 production, as did substitution of the α-amino group with a keto function (to form 3-phenylpyruvic acid). Glycine gave no stimulatory effect, suggesting an essential role of the side chain of D-amino acids for the stimulation. Therefore, in the next experiment, the correlation between the side-chain structure of D-amino acids and the effectiveness on the stimulation was examined with a number of D-amino acids (Table 2). A distinct effect of the side chain in the stimulation of C_2H_4 production was observed between glycine and D-alanine. The stimulatory effect increased, continuously but slightly, as the side chain of D-amino acids was lengthened. On the other hand, branching in the side chain resulted in a more marked increase of the stimulatory effect, e.g. introduction of methyl or phenyl groups at the β-carbon of D-amino acids (to form D-valine, D-isoleucine and D-phenylalanine). Replacement with a hydroxyl group of the methyl group of D-2-aminobutyric acid and D-valine (to form D-serine and D-threonine) decreased the stimulatory effect. Also introduction of a hydroxyl group into the phenyl ring of D-phenylalanine (to form D-tyrosine) decreased the effectiveness of D-phenylalanine. D-Amino acids with negatively or positively charged groups in the side chain, i.e. D-aspartic acid, D-glutamic acid, D-ornithine and D-arginine, had no effect. D-Cysteine severely inhibited C_2H_4 production by the segments, but the inhibition might be due to its action as an SH-compound rather than as an amino acid [7]. D-Methionine and D-ethionine had marked stimulatory effects on C_2H_4 production, probably because of the hydrophobic nature of their side chains. It is thus possible that D-amino acids are effective in the stimulation of C_2H_4 production when their side chains are hydrophobic as well as bulky.

These results indicate that the presence of free α-amino and α-carboxyl groups and of the side chain of amino acids, as well as their D-configurations, is a prerequisite of the stimulation of C_2H_4 production in the segments. By analogy with the three-point contact theory put forward by Easson and Stedman [8] to account for the physiological function of optical isomers, it may be that the initial action of D-amino acids is to specifically interact by means of three of the substituents of their α-carbon atoms, with either some cellular constituent or, possibly, a membraneous receptor site.

Kinetic analysis

Fig. 1 shows dose–response curves for the stimulation of C_2H_4 production by various D-amino acids. The D-amino-acid-stimulated C_2H_4 production was saturated at

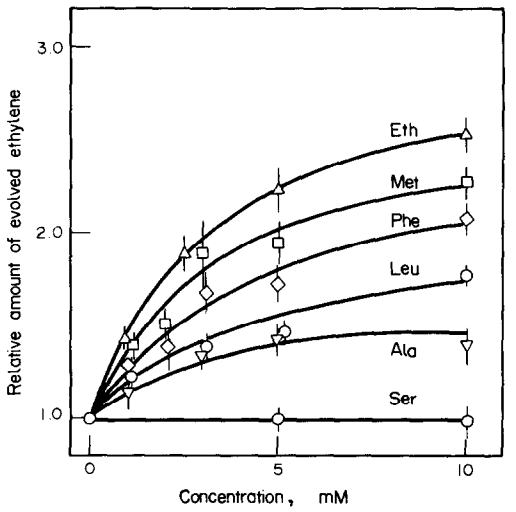

Fig. 1. Dose–response curves for various D-amino acids of C_2H_4 formation by cotyledonary segments. Twenty presoaked segments were incubated with increasing concentrations of D-amino acids at 23° in the dark for 6 hr. Each point represents the mean (±s.e. for 3 replicates). The absolute value for the water control was 0.033 nl/hr/segment.

ca 10 mM with all D-amino acids tested, except D-serine. D-Ethionine was most effective, followed by (in order) D-methionine, D-phenylalanine, D-leucine and D-alanine. When the data were plotted as double reciprocal plots of $1/(RA - 1)$ against 1/[D-amino acid], all the amino acids gave straight lines and had a common abscissa intercept, which means that the acids have the same dissociation constant (K_d = 5.6 mM) for a possible D-amino-acid receptor site. In other words, the affinities of these D-amino acids to the receptor site are the same regardless of difference in side-chain structure. Accordingly, the differences in the effectiveness of various D-amino acids shown in Table 2 are thought to reflect so-called intrinsic activities of the individual side chain of D-amino acids [9].

Although L-phenylalanine itself had no effect on C_2H_4 production in the segments (Table 1), it could competitively inhibit D-phenylalanine-stimulated C_2H_4 production when it was simultaneously applied to the segments with D-phenylalanine (D-phenylalanine, 2, 3, 5 and 10 mM; L-phenylalanine, 2 and 5 mM). The inhibitor constant K_i for L-phenylalanine was calculated as *ca* 5 mM. Similarly, D-serine, which exerted no stimulatory effect on C_2H_4 production by itself (Table 2, Fig. 1), inhibited D-phenylalanine-stimulated C_2H_4 production in a competitive manner on its simultaneous application with D-phenylalanine (D-phenylalanine, 2, 3, 5 and 10 mM; D-serine, 5 mM). K_i for D-serine was also estimated as *ca* 5 mM. These kinetic results can be explained on the assumptions that D-amino acids interact with the possible receptor site in cells and that L-phenylalanine and D-serine compete with D-phenylalanine for that site.

It is very likely, therefore, that D-amino acids cause their stimulatory effect on C_2H_4 production through their interaction with some receptor site in cells, which acts as a trigger reaction for the stimulation of C_2H_4 production. Exceptions, however, are observed in the cases of D-tryptophan and D-asparagine which stimulate C_2H_4 production in a different way to the D-amino acids used in this study (details will appear elsewhere). The site in cells for C_2H_4 synthesis is still obscure. However, the involvement of cell membranes in C_2H_4 synthesis system has been reported [10, 11]. It may be fruitful to seek further evidence for a D-amino-acid receptor site in cell membrane fractions, since the result of such studies may give us a clue to understanding the site for C_2H_4 synthesis in cells.

EXPERIMENTAL

Materials and incubation conditions. Fully after-ripened lower seeds of cocklebur (*Xanthium pennsylvanicum* Wallr.) were used. The preparation and incubation of cotyledonary segments from the seeds have been described previously [5]. Briefly, 20 segments presoaked at 23° in the dark for 24 hr were incubated on a sheet of filter paper (4 cm × 4 cm) wetted with 1 ml of test soln in a 30 ml glass vial under the same conditions. Each incubation was carried out with 3 replicate vials. After incubation for 6 hr, during which time the rate of C_2H_4 production was checked to see if it was constant ±D-phenylalanine (i.e. the stimulation by D-phenylalanine appeared without lag), a 1 ml gas sample was withdrawn with a syringe from the sealed vial and assayed by GSC for C_2H_4. The amounts of C_2H_4 evolved from the segments incubated with chemical solns were normalized against that from a H_2O control (*ca* 0.03 nl/hr/segment) and expressed as the relative amount of evolved C_2H_4 (RA).

Kinetic analysis. C_2H_4 production in the segments in response to exogenously applied D-amino acid was formally treated by the enzyme kinetics of Michaelis and Menten according to the procedure and assumption used by Foster *et al.* [12] in the study of the elongation of *Avena* coleoptile sections in response to exogenous auxin. Data were plotted as double reciprocal plots of $1/(RA - 1)$ against 1/[D-amino acid] and K_d (dissociation constant) calculated by the negative reciprocal of the abscissa intercept of straight lines. In the case of the competitive inhibition, K_i (inhibitor constant) was calculated from the relationship $K_i = \frac{i}{(K_p/K_d) - 1}$ [13], where $K_p = K_d$ in the presence of L-phenylalanine or D-serine at concn *i*.

Chemicals. Chemicals were dissolved in distilled H_2O. The solns of organic acids, amines and acidic or basic amino acids were neutralized with NaOH or HCl before use.

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