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INHIBITION OF PHENYLALANINE AMMONIA-LYASE BY CINNAMIC ACID DERIVATIVES AND RELATED COMPOUNDS

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Abstract—Thirty-five derivatives of cinnamic acid and related compounds were tested for inhibition against phenylalanine ammonia-lyase (PAL) derived from sweet potato, pea and yeast. Caffeic and gallic acids showed inhibition against PAL originating from higher plants, but not against yeast PAL. In contrast, yeast PAL was specifically inhibited by *p*-hydroxycinnamic and *p*-hydroxybenzoic acids. The results suggest that caffeic and gallic acids may act as regulatory substances in phenylpropanoid metabolism in higher plants. Inhibition experiments with synthetic cinnamic acid derivatives have revealed that the presence of a hydrophobic aromatic ring, α,β-double bond and carboxyl group is essential for inhibitory activity. 2-Naphthoic acid which fulfills these structural requirements showed a strong inhibition. The size and shape of the active site is discussed from structure–activity relationships of cinnamic acid derivatives. *o*-Chlorocinnamic acid, one of the strongest inhibitors found in this study, showed an inhibitory effect on the growth of the roots of rice seedlings.

INTRODUCTION

Since Koukol and Conn [1] found phenylalanine ammonia-lyase (PAL) (EC 4.1.3.5) in higher plants, the physiological function of this enzyme has been the subject of extensive studies [2]. Much attention has been paid to its role in the secondary metabolism of plant phenolics [3]. Cinnamic acid and its hydroxyl derivatives were found to be strong inhibitors against PAL [4] and their inhibitory activity was discussed in term of regulatory compounds in secondary metabolism. On the other hand cinnamic acid itself was identified as an allelochemical responsible for allelopathy in guayule [5] and aromatic acids such as *p*-hydroxycinnamic, ferulic and salicylic acids were reported to act as plant growth inhibitors [6, 7]. These facts suggest a possible correlation between plant growth and PAL inhibitory activities. Jangaard [8] investigated the development of herbicides based on PAL inhibition. This paper describes the results of systematic investigations on PAL inhibitory activity of natural aromatic acids and synthetic cinnamic acid derivatives.

RESULTS

Three PAL preparations of different origins were used in this study. Sweet potato PAL induced by cutting was used as a PAL preparation responsible for the biosynthesis of chlorogenic acid [9]. Pea PAL partially purified from pea pods was a preparation relating to the biosynthesis of flavonoids, which was induced by treatment with cuprous chloride [10]. Yeast PAL possessing tyrosine ammonia-lyase (TAL) activity was a commercial enzyme prepared from *Rhodotorula glutinis* [11]. PAL inhibitory activities of aromatic acid which were determined at 1 mM concentration for both substrate and inhibitors are summarized in Table 1. Cinnamic acid was the strongest inhibitor among natural compounds so far tested and showed inhibition against the three kinds of PAL preparations. Gallic and chlorogenic acids, phenolics widely distributed in higher plants, showed inhibition only against PAL preparations derived from higher plants, but not against yeast PAL. Kinetic studies have revealed that gallic acid is a competitive inhibitor of phenylalanine (Fig. 1), whereas chlorogenic acid showed a partially competitive mode in the Dixon plot (Fig. 2). Caffeic and protocatechuic acids that possess a 3,4-dihydroxyphenyl ring system exhibited specific inhibition against sweet potato PAL, but not against pea PAL. By contrast, yeast PAL was inhibited by all the natural cinnamic acid derivatives, as well as by *p*-hydroxybenzoic acid. Inhibition by *p*-hydroxyaromatic acids is a characteristic of yeast PAL having TAL activity.

In order to clarify the structure–activity relationship of PAL inhibition in cinnamic acid derivatives, compounds listed in Table 2 were prepared and tested against sweet potato and yeast PAL. In sweet potato PAL, the inhibitory activity of synthetic derivatives of cinnamic acid, which are substituted in the benzene ring, are in the order chloro, hydroxy and methyl, whereas substitution by a methoxyl group which has a much larger Van der Waals radius tends to decrease the inhibitory activity (Table 2).

Inhibitory activity is also strongly affected by the position of substitution. In general, inhibitory activity decreases in the order *ortho*, *meta* and *para*. In contrast, the effect of substituted groups and positions were quite different in yeast PAL. The decrease of inhibitory activity was only observed in *p*-methylcinnamic acid.

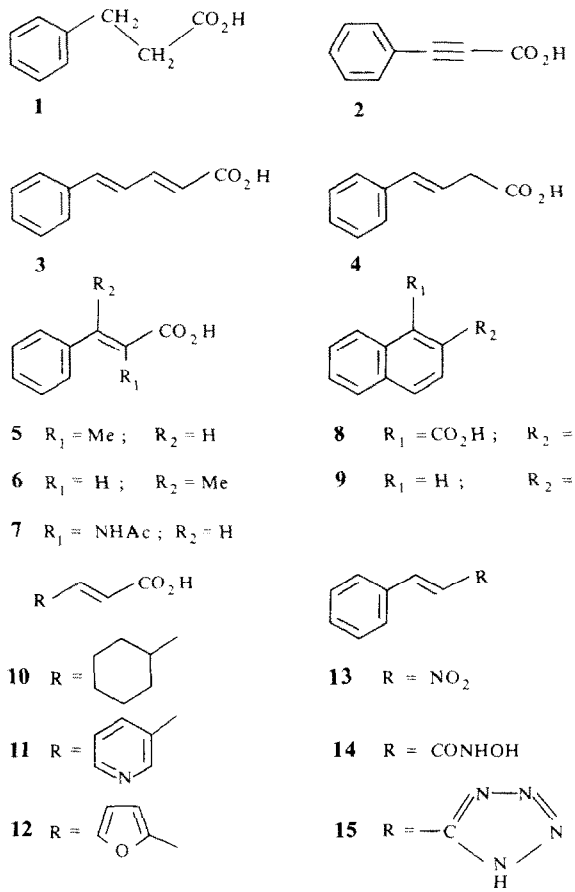

Table 3 summarizes the results of inhibition experiments with compounds of modified cinnamic acid structures. Phenylpropionic acid that lacks an α,β-double bond showed weak inhibition both for sweet potato and yeast PAL. Considerable activity was observed by 4-phenyl-3-butenoic acid, whereas 5-phenyl-2,4-pentadienoic acid showed negligible activity. Introduction of a methyl group at the α- or β-position of cinnamic acid resulted in a lowering of the inhibitory activity and saturation of the benzene ring caused *ca* 50% decrease in activity. When the benzene ring was replaced by hydrophilic aromatic rings such as pyridine or furan, inhibitory activity was lost completely. Compounds possessing nitro, hydroxamate and tetrazole groups instead of carboxylic acid were tested; only nitrostyrene, however,

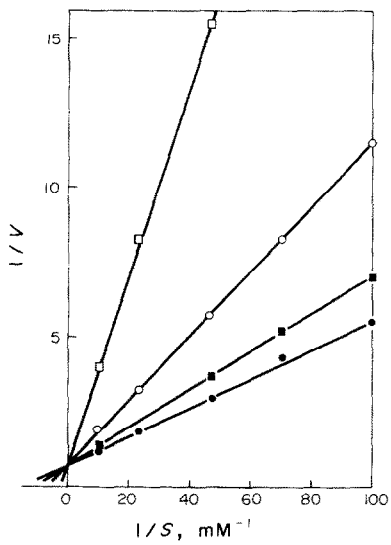

Fig. 1. Lineweaver-Burk plot for sweet potato PAL at different concentrations of gallic acid. ●—●, Without gallic acid; ■—■, 25 μM; ○—○, 100 μM; □—□, 2 mM.

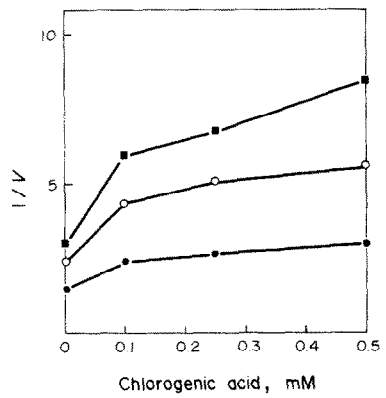

Fig. 2. Dixon plot for sweet potato PAL at different concentrations of substrate (L-phenylalanine). ■—■, 14.3 μM; ○—○, 21.4 μM; ●—●, 42.8 μM.

Table 1. PAL inhibitory activity of phenolic acids

Compound	PAL inhibition (%)		
	Sweet potato	Pea	Yeast
Cinnamic acid	70	74	91
p-Hydroxycinnamic acid	4	3	87
Caffeic acid	62	18	54
Ferulic acid	5	9	87
Benzoic acid	21	22	5
p-Hydroxybenzoic acid	3	6	34
Protocatechuic acid	44	6	2
Gallic acid	72	60	3
Chlorogenic acid	52	34	2

Table 2. PAL inhibitory activity of ring-substituted cinnamic acids

Compound	PAL inhibition (%)	
	Sweet potato	Yeast
Cinnamic acid	70	87
p-Methylcinnamic acid	7	12
m-Methylcinnamic acid	31	77
o-Methylcinnamic acid	77	73
p-Methoxycinnamic acid	1	23
o-Methoxycinnamic acid	5	16
p-Hydroxycinnamic acid	0	85
m-Hydroxycinnamic acid	19	84
o-Hydroxycinnamic acid	74	97
p-Chlorocinnamic acid	21	74
m-Chlorocinnamicacid	63	89
o-Chlorocinnamic acid	84	96
2,6-Dichlorocinnamic acid	26	29

Table 3. PAL inhibitory activity of cinnamic acid analogues

Compound	PAL inhibition (%)	
	Sweet potato	Yeast
Cinnamic acid	70	87
Phenylpropionic acid (**1**)	12	25
Phenylpropiolic acid (**2**)	96	94
5-Phenyl-2,4-pentadienoic acid (**3**)	5	9
4-Phenyl-3-butenoic acid (**4**)	36	48
α-Methylcinnamic acid (**5**)	34	27
β-Methylcinnamic acid (**6**)	41	63
α-*N*-Acetylcinnamic acid (**7**)	52	10
1-Naphthoic acid (**8**)	12	4
2-Naphthoic acid (**9**)	87	71
Cyclohexaneacrylic acid (**10**)	29	33
3-Pyridineacrylic acid (**11**)	6	4
2-Furanacrylic acid (**12**)	2	1
Nitrostyrene (**13**)	76	45
Cinnamohydroxamic acid (**14**)	12	20
5-Styryltetrazole (**15**)	20	40

showed inhibitory activity. It is noteworthy that the inhibition by 2-naphthoic acid was as strong as cinnamic acid, whereas 1-naphthoic acid showed no effect upon PAL activity.

DISCUSSION

Among the compounds tested for inhibitory activity against PAL, kinetic experiments have revealed that cinnamic acid derivatives and 2-naphthoic acid are competitive inhibitors binding to the active site of PAL. Therefore, the inhibitory activity of these compounds would reflect affinity to the active site. Hanson and Rose [12] proposed a model for the active site of PAL, in which a hydrophobic pocket which interacts with a benzene ring plays an important role. The absence of activity in pyridineacrylic and furnanacrylic acid supports the crucial role of the hydrophobic pocket in accommodating a benzene ring. The size of the hydrophobic pocket can be estimated from the results of inhibition experiments with ring substituted cinnamic acid derivatives. Cinnamic acid derivatives possessing a methoxyl group in the benzene ring cannot be accommodated in the hydrophobic pocket, while methyl and chloro groups do not interfere with the hydrophobic interaction in the pocket. The effect of *para* substituents for the inhibitory activity against yeast PAL is particularly informative regarding the property of the hydrophobic pocket. The presence of a methyl at the *para* position markedly suppresses the activity, whereas the effect of hydroxy or chloro groups are not so strong as methyl.

This difference may be due to the difference in the electronic nature of substituent groups, i.e. hydroxy and chloro groups possess lone electron pairs and act as electron-donating groups, whereas the methyl group is proton donating by hyperconjugation. A small electronic change would have a strong effect, since water molecules are not present in the hydrophobic pocket of the substrate binding site.

In sweet potato PAL, the inhibitory activity of ring substituted cinnamic acid derivatives decreases in the order *ortho*, *meta* and *para* substitution, suggesting that the hydrophobic pocket is not large enough to bind strongly with the aromatic ring with *p*-substituents, whereas yeast PAL possesses a somewhat wider pocket and an electrophilic group at the position corresponding to *para* on the benzene ring. The difference between sweet potato and yeast PAL may reflect their different physiological functions. Yeast PAL acts to supply carbon and nitrogen [13] from phenylalanine and tyrosine so that its substrate specificity is lower than PAL of higher plant origin. In contrast, sweet potato PAL produces cinnamic acid leading to caffeic acid [14], a component of chlorogenic acid [15], and the inhibitory action of caffeic and protocatechuic acid may indicate their role in phenolic regulation in sweet potato.

Phenylpropionic and phenylpropiolic acids showed a sharp contrast. The latter is a stronger inhibitor than cinnamic acid, while the former acts as a very weak inhibitor, indicating an essential requirement for the presence of π electrons in α- and β-positions. This is further supported by the strong inhibitory action of 2-naphthoic acid, which fulfills the necessary requirements as a strong inhibitor. 2,6-Dichlorocinnamic acid showed much lower inhibition compared to *o*-chlorocinnamic acid, the former compound being different in lacking planarity [16].

The length of the molecule is also important for inhibitory activity. Results observed with synthetic cinnamic acid derivatives support the active site model proposed by Hanson *et al.* [17]. The narrow hydrophobic pocket which accommodates the benzene ring and a cationic site to trap carboxylate are present in the crevice-shaped active site and the length of the crevice is approximately the same as that of the cinnamate molecule. Yeast PAL differs in the shape of its hydrophobic pocket. It can accommodate a benzene ring substituted with chloro or methyl groups, and the electrophilic group is present at the position corresponding to the *para* of the benzene ring. It has been pointed out that the active site of PAL can take two conformations [17]; of these, the conformation binding with cinnamic acid, the reaction product, is more stable [14]. This may be the reason for phenylpropiolic and 2-naphthoic acids showing strong inhibition.

Cinnamic acid derivatives were tested for inhibitory effect against rice shoot growth (Table 4). Growth inhibition of rice shoot roots by cinnamic acid derivatives suggests that it is caused by the inhibition of PAL. However, since cinnamic acid itself showed strong growth inhibition, one cannot conclude that this is caused by the inhibition of lignification because cinnamic acid was supplied exogenously to be utilized in lignification. It would be reasonable therefore to assume that it is caused by the inhibition of phenylalanine metabolism.

The results so far obtained in the inhibition studies with aromatic acids indicate that the first enzyme of the phenylpropanoid metabolism, PAL, may be affected by phenolic acids formed in the pathway. This does not necessarily mean that the aromatic acids act as regulators specific for PAL activity in plants, since K_i values of the inhibitors are too high, i.e. the same order as the K_m values (Table 5). In general, the content of phenolics in higher plants is relatively high and there remains the possibility that together they control PAL activity.

Table 4. Growth inhibitory activity of PAL inhibitors on rice seedlings

Sample	Length (mm)	
	Root	Leaf sheath
Control	41 ± 4	29 ± 4
Cinnamic acid		
0.1 mM	39 ± 4	28 ± 4
1.0 mM	4 ± 2	22 ± 4
p-Hydroxycinnamic acid		
0.1 mM	39 ± 9	28 ± 2
1.0 mM	32 ± 7	27 ± 1
o-Chlorocinnamic acid		
0.1 mM	19 ± 4	28 ± 3
1.0 mM	4 ± 2	19 ± 3

Table 5. Inhibition constants of PAL inhibitors

Enzyme source	Inhibitor	K_i (M)
Sweet potato	Cinnamic acid	1.6×10^{-5}
K_m 6.3×10^{-5} M	Gallic acid	8.8×10^{-5}
	2-Naphthoic acid	1.0×10^{-5}
	o-Chlorocinnamic acid	1.2×10^{-5}
Yeast	Cinnamic acid	3.8×10^{-5}
K_m 3.1×10^{-4} M	Ferulic acid	8.0×10^{-5}
	p-Hydroxybenzoic acid	1.8×10^{-5}

EXPERIMENTAL

Chemicals. Methyl, methoxy and chloro-derivatives of cinnamic acids, and 2-furnanacrylic and 3-pyridineacrylic acids were synthesized from the corresponding aldehydes by the Knoevenagel reaction with Doebner's modification [18]. 4-Phenyl-3-butenoic and α-methylcinnamic acids [19, 20] were prepared by the Perkin reaction and β-methylcinnamic acid [21], nitrostyrene [22], cinnamohydroxamic acid [23] and 5-styryltetrazole [24] by known procedures. Phenylpropiolic and α-*N*-acetylcinnamic acids were kind gifts of Professor K. Koga of the Faculty of Pharmaceutical Sciences, University of Tokyo. Chlorogenic acid was donated by Dr. A. Komamine, Department of Botany, University of Tokyo. The other chemicals were purchased from Tokyo Kasei Co. Ltd.

Enzyme preparations. Sweet potato PAL was prepared according to the procedure of Tanaka and Uritani [25] and purified up to 140-fold. Pea PAL was prepared from pea pods purchased in the market. Pea pods (1 kg) were immersed in 3 mM $CuCl_2$ soln for 17 hr at 20° to induce PAL activity. After this treatment, they were homogenized with a Waring blender in 0.1 M borate buffer (pH 8.5) containing 2-mercaptoethanol (0.8 ml/l), 1 mM EDTA, 50 mM potassium isoascorbate and filtered through four folds of linen. The filtrate was centrifuged at 7000 *g* for 15 min and the supernatant further purified according to the method used for the preparation of sweet potato PAL. The enzyme was purified up to 20-fold. Yeast PAL originating from *Rhodotorula glutinis* was purchased from P.L.-Biochemical Inc., U.S.A.

Assay of PAL activity. The standard reaction mixture (1 ml) contains 1 mM L-[U-^{14}C]phenylalanine, 50 mM Tris–HCl (pH 8.5), enzyme preparation, and 50 μl of an ethyleneglycol monomethyl ether (EGME) soln of inhibitor. The addition of EGME up to 10% had no effect upon the reaction. The reaction mixture was incubated at 40° for 15 min and the reaction stopped by the addition of 6N HCl (100 μl). Toluene (2 ml) was added to the reaction mixture, which was thoroughly mixed with a Voltex mixer. A 1 ml aliquot of toluene was counted in toluene liquid scintillator cocktail. Percentage inhibition was calculated from the decrease of radioactivity in the presence of inhibitor.

Plant growth inhibition test with rice seedlings. The tests were carried out according to the procedure of Sato [26]. Four rice seedlings germinated in distilled H_2O were fixed onto a plastic disk and put in a plastic container containing a 5% EtOH soln (pH 8–9) of test sample. The seedlings were incubated for 4 days in a chamber with 12 hr alternate light (fluorescent light 3000 lx) and darkness. Eight seedlings were used for one sample and the mean lengths of leaf sheaths and main roots are presented in Table 5.

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