Enzymes in Metabolism of Amino Acids. VII.* Phenylalanine Ammonia-Lyase in Maize (*Zea mays* L.)

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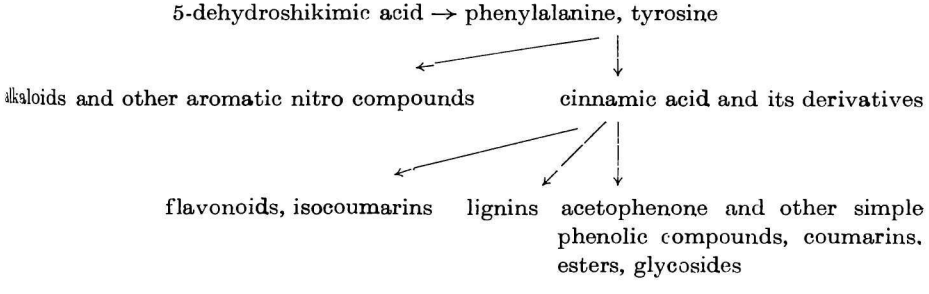
We have isolated an enzyme, phenylalanine ammonia-lyase (EC 4.3.1.5.), from the rootlets of *Zea mays* var. *CE-IV*. After partial purification by fractional salting out with ammonium sulfate and gel chromatography on Sephadex G-100, we obtained a preparation of a 40-fold greater specific activity than that of the crude homogenate. We have determined the apparent Michaelis constant and the activation energy of this purified preparation and investigated the effect of some phenolic compounds on its activity.

The reaction catalyzed by phenylalanine ammonia-lyase (EC 4.3.1.5.) connects the aromatic amino acids with biosynthesis of phenolic compounds in plants. In this reaction, phenylalanine is deaminated to *trans*-cinnamic acid [1]. Further hydroxylation and methylation reactions give *p*-coumaric, caffeic, ferulic, and sinapic acids which participate in the formation of lignin, polyphenols (as coumarins, flavonoids) and many other more complicated phenolic compounds [2–4].

The mentioned deamination reaction might have a key position regarding the regulation of biosynthesis of all compounds which follow after cinnamic acid in the biogenetic sequence as it is shown in Scheme 1.

Scheme 1



Phenylalanine ammonia-lyase was proved in a great number of venosal plants [5] and in some basidial fungi [6]. The question of reversible regulation of phenylalanine ammonia-lyase activity by some phenolic compounds was studied mainly with preparations obtained from tobacco leaves (*Nicotiana tabacum* L.) [7].

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We have studied the effect of hydroxy derivatives of cinnamic acid and some coumar derivatives on the activity of phenylalanine ammonia-lyase isolated from the rootlets maize.

Experimental

The source of enzyme were the main rootlets of three days germinated *Zea mays* va *CE-IV*. Seeds that had been soaked 12 hours in water were germinated on a moisten polymeric urethan support in the dark at 25°C.

The separated rootlets were homogenized by pulverizing with sand in 0.05 M phospha buffer of pH 7. The homogenate was centrifuged at 10,000 *g* for 20 minutes (Centrifu Janetzki K-50). The supernatant was fractionated with ammonium sulfate [9]. Th protein fraction obtained by saturating the solution to 20—60% was dissolved in 10 m of 0.005 M phosphate buffer, dialyzed for 16 hours against 4000 ml of 0.001 M phospha buffer of pH 7 and separated on a column (3 × 100 cm) of Sephadex G-100. Phospha buffer of pH 7 was used as eluent. At a flow rate of 6 ml h^{-1}, 3-ml fractions were collecte from the column. The combined fractions No. 35—45 were used as enzyme preparatio All operations during isolation and purification of the enzyme were performed at 0°C.

The activity of phenylalanine ammonia-lyase was determined by spectrophotometri measurement of *trans*-cinnamic acid in the incubation mixture [1].

The incubation mixture contained 0.5 ml of enzyme preparation, 20 μmoles of L-phenyl alanine and was adjusted to 3 ml volume by 0.2 M Tris-HCl of pH 8.8. After 60 minute of incubation, 0.5 ml of the incubation mixture was taken, made up to 3 ml with 0.5 sodium hydroxide and its absorbance measured at 268 nm. A blank solution was th sample taken at zero time of incubation.

The concentration of the product increased in the range from 0 to 60 minutes linearl at the incubation temperature 40°C. To obtain higher values of absorbances, we hav chosen 60 minutes for the incubation.

The values of cinnamic acid concentration were read off on the calibration curve.

The content of proteins was determined by *Lowry* method [8].

The presence of *p*-coumaric acid was determined by thin-layer chromatograph performed in the system benzene—methanol—formic acid (50 : 2 : 1). Layers of Kieselge H (Merck) of 0.3 mm thickness on plates (50 × 200 mm) activated for 3 hours at 110° were used. Compounds were detected with molybdophosphoric acid.

The unit of the enzyme activity (U) was defined as that quantity of enzyme whic catalyzed the conversion of 1 μmole of phenylalanine to 1 μmole of cinnamic acid per on minute.

Results and Discussion

The separation of the proteins and the phenylalanine ammonia-lyase activity on th column of Sephadex G-100 is apparent from Fig. 1. The results obtained in individua steps of purification are in Table 1.

The dependence of phenylalanine ammonia-lyase activity on pH of the medium an on temperature was described already [9]. The partly purified enzyme was used t investigate the kinetic data of the reaction under study. We have determined the apparen Michaelis constant after *Lineweaver* and *Burk* [10]. For L-phenylalanine in the concentra tion range from 3.3×10^{-4} M to 2×10^{-2} M at pH 8.8 $K_m = 4 \times 10^{-3}$ M. The value o activation energy was 12,763 cal mol^{-1} in the range from 35 to 45°C. The thermal coef ficient Q 10 for the same thermal range was 1.94.

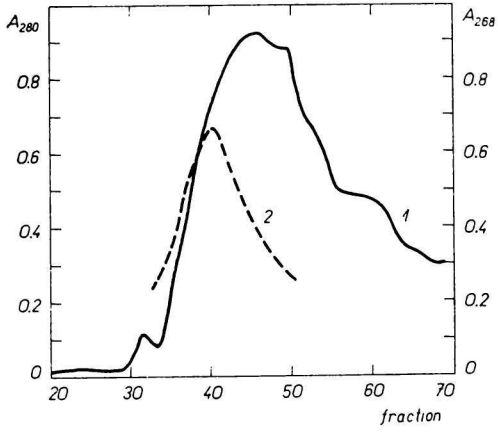

Fig. 1. Chromatographic separation of proteins and phenylalanine ammonia-lyase activity on Sephadex G-100.
A_{280} – concentration of proteins expressed by absorbance at 280 nm; A_{268} – enzyme activity expressed by absorbance of the incubated mixture at 268 nm.
1. proteins; *2.* enzyme activity.

Table 1

Purification of phenylalanine ammonia-lyase

Step of purification	Volume [ml]	mU	Proteins [mg]	Specific activity (mU mg P^{-1})	Increase in specific activity (fold)
crude homogenate	80	2500	2100	1.19	1
dialysate	15	880	205	4.29	3.6
enzyme preparation	20	740	15.5	47.7	40.2

The value of apparent Michaelis constant found in our experiments does not differ very much from that found for the enzyme isolated from barley which was 1.3×10^{-3} M [1]. However, for individual enzymes isolated from different sources different K_m values were found. *O'Neal* [7] confirmed that it could be explained by different affinity of the enzyme to the substrate because of different phenylalanine concentration in the appropriate plants.

When replacing phenylalanine with tyrosine, we found that tyrosine was not deaminated by partly purified enzyme or crude homogenate.

We have investigated the effect of some phenolic compounds on the activity of phenylalanine ammonia-lyase with the aim to find out the possibilities of regulation of this reaction by feed-back inhibition with compounds participating in the biosynthesis of more complex phenolic compounds. The obtained results are summarized in Table 2.

Fig. 2 shows that it is the competitive inhibition in all cases.

Table 2

Effect of different inhibitors on the activity of phenylalanine ammonia-lyase
Concentration of L-phenylalanine 6.6×10^{-3} M

Inhibitor	Concentration of the inhibitor [M]	Relative activity [%]
p-coumaric acid	1.66×10^{-4}	28.6
caffeic acid	1.66×10^{-4}	53.5
ferulic acid	1.66×10^{-4}	100.0
chlorogenic acid	1.66×10^{-4}	85.6
scopoletin	1.66×10^{-4}	90.4
L-tyrosine	9.37×10^{-4}	24.2
potassium cyanide	5.00×10^{-3}	29.3
control	—	100.0

The values of K_i for the used inhibitors are: 8.6×10^{-5} M for *p*-coumaric acid, 2.4×10^{-4} M for caffeic acid, 1.6×10^{-3} M for chlorogenic acid, 7.8×10^{-3} M for scopoletin and 1.2×10^{-3} M for L-tyrosine.

The remarkable inhibition effect of sodium cyanide is in agreement with the result obtained with enzyme isolated from potatoes [11] and tobacco leaves [7]. The author explained this effect by the presence of carbonyl group in the active centre of the enzyme

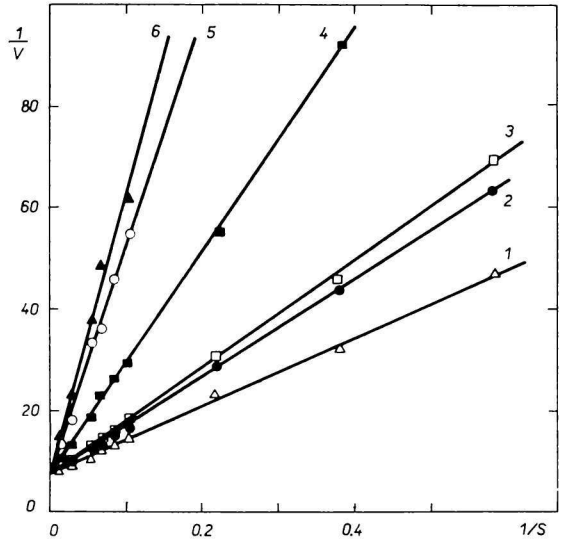

Fig. 2. The reciprocal values of reaction rate in dependence on the reciprocal values of substrate concentration.
1. without inhibitor; *2.* scopoletin; *3.* chlorogenic acid; *4.* caffeic acid; *5. p*-coumaric acid; *6.* L-tyrosine.

which was not identical with the aldehyde group of pyridoxal phosphate. Recently *Havir* and *Hanson* [12] have studied the mechanism of deamination of phenylalanine and proved the presence of dehydroalanyl residue as prosthetic group in the active centre of phenylalanine ammonia-lyase.

It is interesting to compare the effect of some phenolic compounds on the activity of phenylalanine ammonia-lyase isolated from other sources than maize. The enzyme isolated from tobacco leaves is not sensitive to the presence of *p*-coumaric, caffeic, and chlorogenic acid. On the other hand, it is influenced by ferulic acid which does not affect the activity of the maize enzyme. Two isoenzymes of phenylalanine ammonia-lyase were isolated from oak leaves [13] which differed by sensitivity to phenolic compounds but were inhibited by the same compounds as the enzyme from maize.

Though different sensitivity of phenylalanine ammonia-lyase (from different sources) to the derivatives of cinnamic acid has been found, the function of these compounds as regulators of cinnamic acid formation is apparent. The level of phenolic compounds in organism is thus directly dependent on the presence of one of the enzymes of their biosynthesis — phenylalanine ammonia-lyase and the appropriate hydroxylases. These enzymes are regulated by primary exogeneous factor — light [14] — which induces their de novo synthesis and thus the increase of phenolic compounds in organism. On account of that the growth itself is influenced; phenolic compounds control the activity of indolylacetic acid oxidase which determines the endogeneous concentration of the growth hormone — indolylacetic acid. From our experimental results we can assume that phenols influence also the activity of phenylalanine ammonia-lyase and thus cause the decrease of cinnamic acid formation and regulate their own concentration in organism.

The obtained results contribute to the recognition of the mode of formation and changes of amino acids which are related to biosynthesis of the discussed compounds in plants.

References

1. Koukol J., Conn E. E., *J. Biol. Chem.* ***236***, 2692 (1961).
2. Brown S. A., Neish A. C., *Can. J. Biochem. Physiol.* ***33***, 948 (1955).
3. Brown S. A., Neish A. C., *Can. J. Biochem. Physiol.* ***34***, 1037 (1958).
4. Bland D. E., Logan A. F., *Phytochemistry* ***6***, 1075 (1967).
5. Young M. R., Towers G. H. N., Neish A. C., *Can. J. Biochem. Physiol.* ***44***, 341 (1966).
6. Bandoni R. J., Moore K., Subba Rao P. V Towers G. H. N., *Phytochemistry* ***7***, 205 (1968).
7. O'Neal D., Keller C. J., *Phytochemistry* ***9***, 1373 (1970).
8. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.* ***193***, 265 (1951).
9. Pšenáková T., Kovács P., Pšenák M., *Phytochemistry* ***9***, 2277 (1969).
10. Lineweaver H., Burk D., *J. Amer. Chem. Soc.* ***56***, 568 (1934).
11. Havir E. A., Hanson K. R., *Biochemistry* ***7***, 1896 (1968).
12. Hanson K. R., Havir E. A., *Arch. Biochem. Biophys.* ***141***, 1 (1970).
13. Boudet A., Ranjeva R., Gadal P., *Phytochemistry* ***10***, 997 (1971).
14. Zucker M., *Plant Physiol.* ***40***. 779 (1965).

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