Biosynthesis of Alkaloids. XXVIII.* L-Phenylalanine:2-Oxoglutarate Aminotransferase from *Lobelia inflata* L. Plants

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Received July 15, 1971

From extracts of the aerial parts of *Lobelia inflata* L. plants, a 100-fold purified preparation of L-phenylalanine:2-oxoglutarate aminotransferase (EC 2.6.1.5.) was obtained by salting out with ammonium sulfate and chromatography on Sephadex G-75 and G-200. The optimal activity of the enzyme was in the pH range 8.0—9.0. The apparent Michaelis constant for L-phenylalanine was 5.3×10^{-2} M and for 2-oxoglutarate 4.6×10^{-5} M. The enzyme catalyzed the transamination not only of phenylalanine but also of other aromatic and aliphatic amino acids in the presence of 2-oxoglutarate as amino group acceptor. The most active amino group donors seemed to be L-aspartic acid and asparagine.

One of the basic precursors for biosynthesis of lobeline in *Lobelia inflata* plants is phenylalanine [1, 2]. Transamination reaction catalyzed by the appropriate aminotransferase (EC 2.6.1.5.) plays a significant role in securing its proper level in plants by transforming phenylpyruvic acid to phenylalanine [3—5].

In connection with the study of lobeline formation in *Lobelia inflata* plants, we followed in our previous work [6] the correlation between the formation of lobeline and activity of phenylalanine aminotransferase during ontogenesis. In order to know more about the properties of this aminotransferase, we partly purified and then characterized this enzyme by kinetic data as well as by study of substrate specificity.

Experimental

Plant material

The seeds of *Lobelia inflata* were sown in the middle of March in a greenhouse. The plants were transplanted at the beginning of May and cultivated under normal soil conditions. The aerial parts of plants in phase of flowering (21st—24th week of growth) were used for experiments.

Determination of enzyme activity

The activity of L-phenylalanine:2-oxoglutarate aminotransferase was evaluated by determining the formed phenylpyruvic acid spectrophotometrically according to *Gamborg* and *Wetter* [7]. The reaction mixture contained 50 μmoles of L-phenylalanine, 2.5 μmoles

* For Part XXVII. see Ref. [1].

of 2-oxoglutarate, 0.1 μmole of pyridoxal-5-phosphate, 50 μmoles of phosphate buffer (Na^+) of pH 8.0, and the enzyme solution corresponding to 180—600 μg of proteins in the final volume (2.0 ml); the temperature was 38°C. The enzyme preparation was preincubated for 30 minutes with the solution of pyridoxal-5-phosphate before adding the substrate. Immediately after adding the substrate, 0.5 ml of the mixture was withdrawn and made up to 2.5 ml with 0.5 N solution of sodium hydroxide. The further samples were taken similarly after 15—60 minutes' incubation and absorbance measured at 320 nm against the control samples (the mixture incubated without exogenous amino acid). The amounts of the formed phenylpyruvic acid were read off on the calibration curve.

The unit of the enzyme activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmole of phenylpyruvic acid per minute under the mentioned experimental conditions (mU = 1 × 10^{-3} U). The specific enzyme activity was expressed in milliunits per 1 mg of proteins (mU mg P^{-1}; P = protein).

Determination of proteins

Proteins were determined spectrophotometrically according to *Warburg* and *Christian* [8]. All spectrophotometric measurements were taken on a Spectromom 202 in 1-cm cells.

Extraction and purification of enzyme

The extraction and purification of L-phenylalanine:2-oxoglutarate aminotransferase were carried out at 0—4°C. The fresh plant tissue (60 g) was disintegrated with sand and extracted two times always with 60 ml of 0.025 M phosphate buffer (Na^+) pH 8.0 for 15 minutes. These extracts were filtered through a silon cloth and the joined filtrates centrifuged at 10,000 *g* for 20 minutes in a Janetzki K-50 cooled centrifuge. The supernatant (denoted as crude homogenate) was fractionated with ammonium sulfate to 0—30, 30—60, and 60—100% saturation. The individual protein fractions were centrifuged at 10,000 *g* for 20 minutes, dissolved in a minimal amount of 0.025 M phosphate

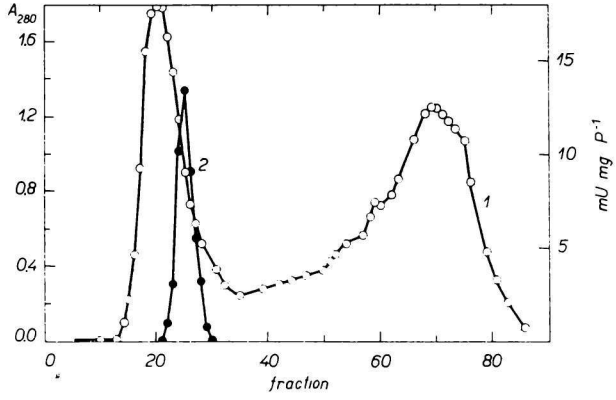

Fig. 1. Elution of proteins (*1*) and specific activity of L-phenylalanine:2-oxoglutarate aminotransferase (*2*) from a Sephadex G-75 column.

buffer pH 8.0 and dialyzed against the same buffer for 20 hours. The precipitates formed during dialysis were centrifuged at 10,000 *g* for 20 minutes and the enzyme activity was determined in supernatants. The fraction obtained from 30—60% saturation with ammonium sulfate was applied to a column of Sephadex G-75 (2.8 × 50 cm) previously equilibrated with 0.025 M phosphate buffer pH 8.0. The proteins were eluted with the same buffer at a rate of 10 ml/hour; 5-ml fractions were collected. The evaluation of proteins in fractions was carried out by measuring the u.v. absorption at 280 nm (Fig. 1).

The fractions 24—26 emerging from Sephadex G-75 were pooled, applied to a column of Sephadex G-200 (2.8 × 35 cm) previously equilibrated with 0.025 M phosphate buffer pH 8.0 and eluted with the same buffer at a rate of 6 ml/hour; 3-ml fractions were collected (Fig. 2).

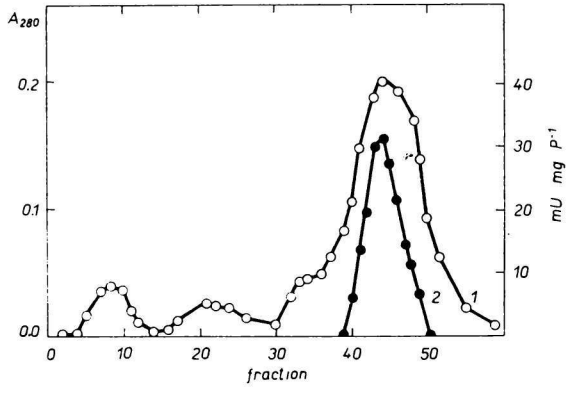

Fig. 2. Elution of proteins (*1*) and specific activity of L-phenylalanine:2-oxoglutarate aminotransferase (*2*) from a Sephadex G-200 column.

Properties of enzyme

pH optimum

The dependence of enzyme activity on pH was followed at pH 6.0—8.0 (0.1 M phosphate buffer), 7.4—9.0 (0.2 M Tris-HCl buffer), and 10.0 (0.1 M carbonate buffer). An enzyme solution of a specific activity 21.7 mU mg P^{-1} obtained from Sephadex G-200 was used for these experiments.

Effect of temperature on enzyme activity and thermal stability of enzyme

The effect of temperature on the enzyme activity was followed under the mentioned experimental conditions using the enzyme of a specific activity 9.2 mU mg P^{-1} from Sephadex G-75. The reaction mixtures were incubated at 20—80°C for 15 minutes.

For thermal stability determination, the enzyme solution of a specific activity 14 mU mg P^{-1} from Sephadex G-200 was used. The enzyme solutions were heated at 30—80°C for 10 minutes, cooled to 0°C, and their activity established after 60 minutes' incubation.

Substrate specificity

To study the substrate specificity, the enzyme solution of a specific activity 22 mU mg P^{-1} from Sephadex G-200 was used. L-Aspartic acid, asparagine, L-leucine, L-lysine, L-methionine, L-ornithine, L-serine, L-tryptophan, L-tyrosine, and L-valine were used as amino group donors and 2-oxoglutarate as acceptor. The concentration of amino acids in the reaction mixture was 2.0×10^{-2} M except that of tyrosine which was 1.0×10^{-2} M. The transamination reactions were evaluated by determining the formed glutamic acid after 90 minutes' incubation in 0.1 ml of the reaction mixture as described in our previous work [6]. The enzyme activity was expressed in μmoles of formed glutamic acid during 90 minutes per 1 mg of proteins.

Results and Discussion

Results of the L-phenylalanine:2-oxoglutarate aminotransferase purification extracted from the aerial parts of *Lobelia inflata* plants (phase of flowering) are presented in Table 1.

For characterization of the enzyme, eluates from Sephadex G-200 and G-75 respectively, were used. The rate of transamination reaction was directly proportional to the enzyme

Table 1

Purification of L-phenylalanine:2-oxoglutarate aminotransferase from the aerial parts of *Lobelia inflata* L.

Mode of purification	Volume [ml]	Proteins [mg]	Activity [mU]	Specific activity [mU mg P^{-1}]	Degree of purification
I. Crude extract	102.0	1271.15	378.0	0.297	1
II. 30—60% $(NH_4)_2SO_4$	15.6	505.75	312.0	0.617	2
III. Sephadex G-75 fraction 25	5.0	6.15	82.9	13.490	45
IV. Sephadex G-200 fraction 44	3.0	0.47	14.7	31.277	105

concentration for 90 minutes under the mentioned experimental conditions. The optimum enzyme activity in phosphate buffer was at pH 8.0; in Tris-HCl at pH 9.0 (Fig. 3). In the pH range 7.4—8.0, the enzyme activity in Tris-HCl was 40—60% lower than that in phosphate buffer. Similar effect of Tris-HCl on the activity of L-phenylalanine:2-oxoglutarate aminotransferase was found also by other authors [9].

The activity of L-phenylalanine:2-oxoglutarate aminotransferase increased up to 55°C (Fig. 4), the activation energy over the range 30—40°C was 17,640 cal mol^{-1}. The enzyme was relatively stable against the inactivation by temperature. After 10 minutes' heating at 55°C, 65% of the original activity retained. Total inactivation of the enzyme appeared only at temperatures higher than 70°C (Fig. 5).

Preincubation of the enzyme with pyridoxal-5-phosphate increased its activity by about 17% in comparison with the control samples (without exogenous cofactor).

The effect of concentrations of L-phenylalanine and 2-oxoglutarate on the reaction rate is shown in Fig. 6. The enzyme activity was determined after 1-hour incubation under the described experimental conditions. The L-phenylalanine concentration in the reaction mixture varied in the range from 1.25×10^{-3} M to 4.0×10^{-2} M at the constant concentration of 2-oxoglutarate (1.25×10^{-3} M). The 2-oxoglutarate concentration

varied in the range from 5.0×10^{-5} M to 1.25×10^{-3} M at the constant concentration of L-phenylalanine (2.5×10^{-2} M). Transamination reaction was inhibited by increased concentration of 2-oxoglutarate ($>3.0 \times 10^{-3}$ M). The apparent Michaelis constant, determined graphically according to *Dixon* and *Webb* [10], was 5.3×10^{-2} M for L-phenylalanine and 4.6×10^{-5} M for 2-oxoglutarate. Also other transaminases were found to have greater affinity to keto acid [9, 11, 12].

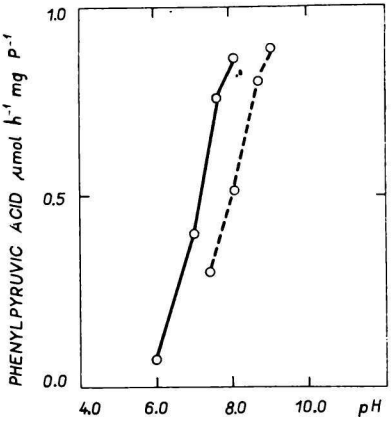

Fig. 3. Effect of pH on the activity of L-phenylalanine:2-oxoglutarate aminotransferase.
● 0.1 M phosphate buffer (Na^+);
○ 0.2 M Tris-HCl buffer;
△ 0.1 M carbonate buffer.

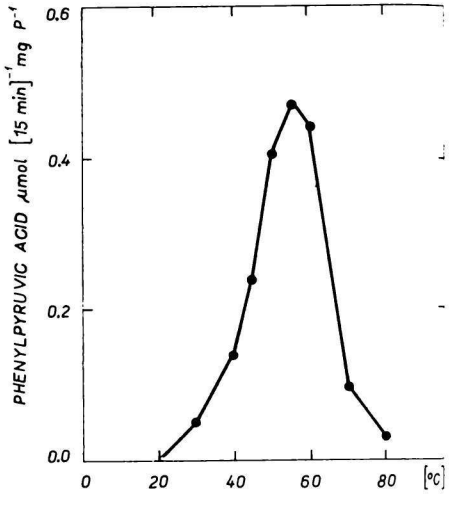

Fig. 4. Effect of temperature on the activity of L-phenylalanine:2-oxoglutarate aminotransferase.

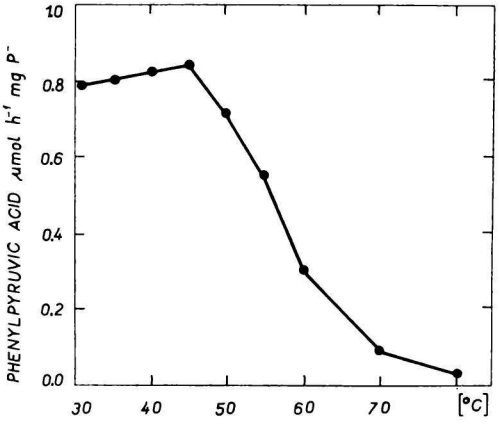

Fig. 5. Thermal stability of L-phenylalanine:2-oxoglutarate aminotransferase.

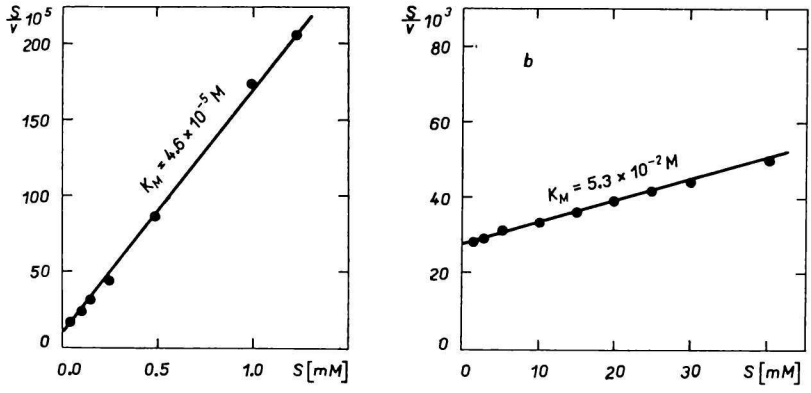

Fig. 6. Graphical determination of K_M for 2-oxoglutarate (*a*) and for L-phenylalanine (*b*). *v* is expressed in μmoles of formed phenylpyruvic acid during 1 hour per 1 mg of proteins.

Table 2

Transamination reaction of amino acids catalyzed by aminotransferase from *Lobelia inflata* L.

L-Amino acid	Activity
aspartic acid	8.79
asparagine	0.86
phenylalanine	0.63
leucine	0.36
lysine	0.00
methionine	0.23
ornithine	0.16
serine	0.00
tryptophan	0.59
tyrosine	0.18
valine	0.18

Purified enzyme preparations catalyzed transamination reactions of aromatic and aliphatic amino acids with 2-oxoglutarate as amino group acceptor (Table 2). The most active amino group donors were L-aspartic acid and asparagine. The rate of transamination reaction of other amino acids, with the exception of L-tryptophan, was substantially lower than that of L-phenylalanine. The problem whether the reaction is catalyzed by one or more specific aminotransferases, will be the subject of further study.

References

1. Šmogrovičová H., Nemec P., Kompiš I., Jindra A., Kovács P., Thurzová S., *Acta Fac. Pharm.* ***17***, 147 (1969).
2. Keogh M. F., O'Donovan D. G., *J. Chem. Soc. C****1970***, 2470.

3. Kretovič V. L., Uspenskaja Ž. V., *Biochimija* ***23***, 248 (1958).
4. Gadal P., Bouyssou H., Barthe J. P., *Physiol. Veg.* ***7***, 69 (1969).
5. Redkina T. V., Uspenskaja Ž. V., Kretovič V. L., *Biochimija* ***34***, 312 (1969).
6. Šmogrovičová H., Jindra A., Kovács P., *Collect. Czech. Chem. Commun.* ***33***, 1967 (1968).
7. Gamborg O. L., Wetter L. R., *Can. J. Biochem. Physiol.* ***41***, 1733 (1963).
8. Warburg O., Christian W., *Biochem. Z.* ***310***, 384 (1941).
9. George H., Gabay S., *Biochim. Biophys. Acta* ***167***, 555 (1968).
10. Dixon M., Webb E. C., *Enzymes*, Longmans, London, 1964.
11. Kagan Z. S., Dronov A. S., Kretovič V. L., *Dokl. Akad. Nauk SSSR* ***179***, 1236 (1968).
12. Dumitru I. F., Iordachescu D., Niculescu S., *Rev. Roum. Biochim.* ***7***, 31 (1970).

Translated by A. Kardošová