Chromatographic Purification, Crystallization and Study of Vegetable L-Alanine: 2-Oxoglutarate-Aminotransferase Properties

Numerous authors demonstrated that aminotransferases are present in vegetable tissues, their activity varying from one species to another or during the growth and development of plants. Thus, CRUICHSHANK and ISHERWOOD[1] studied glutamic-alanine and glutamic-aspartic transaminase from wheat germ. BAPTIST[2], PATWARDHAN[3], DAVIES and ELLIS[4] isolated, by the method of precipitations fractioned with $(NH_4)_2SO_4$ and by chromatography on column, glutamic-oxaloacetic transaminase from cauliflower.

The purpose of the present work consists in chromatographic purification, crystallization and study of certain physical and chemical properties of vegetable L-alanine: 2-oxoglutarate-aminotransferase from *Glycine hispida* seeds.

Material and methods. The extraction of proteins was effected from acetonic powder of Glycine hispida seeds at the temperature of 4 °C, 60 min, with 40 ml KH_2PO_4/K_2HPO_4 $5\times10^{-3}M$, pH 7.5 buffer.

The resulting homogenate was filtered and centrifuged for 30 min at $20{,}000\times g$ and the supernatant was employed for the subsequent enzyme purification.

Protein determination was effected according to the method of LOWRY et al.[5]. Measurement of L-alanine: 2-oxoglutarate-aminotransferase activity, was carried out according to the method of ROSEN et al.[6]. Enzyme specific activity was expressed in EC I.U.B. units: μM pyruvic acid/min/mg protein.

All reagents employed in the present work were furnished by the firms Merck, BDH, Calbiochem and Pharmacia Uppsala.

Total extract was adsorbed on a column of Sephadex G-50 medium (23×2.8 cm) and eluated at a rate of 13 ml/h. Column elution was achieved with a KH_2PO_4/K_2HPO_4 $5\times10^{-3}M$, pH 7.5 buffer solution, and fractions of 10 ml each were collected. Eluates obtained from the column of Sephadex G-50 medium were passed on a column of DEAE-cellulose SS (22×1.8 cm). Elution was achieved by successive introduction of pH 7.5 buffer solutions: 10 ml of $2\times10^{-2}M$, 20 ml of $5\times10^{-2}M$ and 50 ml of $6\times10^{-1}M$ phosphates buffer. A relatively great enzyme activity was detected exclusively in fractions eluated with $6\times10^{-1}M$ potassium phosphate buffer.

These samples were reunited, dialyzed for 24 h against a $5\times10^{-3}M$ potassium phosphate buffer, and the dialysate was introduced on a column of DEAE-cellulose of the hydroxylapatite type (9.5×1.8 cm). The column was eluated with 30 ml of $2\times10^{-2}M$, pH 7.5 phosphate buffer solution and fractions of 2 ml each were collected.

These eluates were introduced on a hydroxylapatite column (12.5×1.8 cm) preparated after TISELIUS, HJERTEN and LEVIN[7]. The hydroxylapatite column was subsequently eluated with solutions of phosphate buffer pH 7.5 of different molarities: 15 ml — $2\times10^{-2}M$, 20 ml — $4\times10^{-2}M$, 15 ml — $8\times10^{-2}M$ and 25 ml — $2\times10^{-1}M$ buffer solutions and eluates of 2 ml each were collected. Proteins with enzyme activity are eluated in $4\times10^{-2}M$ potassium phosphate buffer solution.

Finally, fractions with enzyme activity were introduced on a column of Sephadex G-50 medium equilibrated with NaCl $1\times10^{-1}M$ and the column was eluated with 25 ml of NaCl $1\times10^{-1}M$ solution and 40 ml NaCl $2\times10^{-1}M$ solution. Proteins were detected in eluates obtained after the introduction of NaCl $1\times10^{-1}M$.

Results and discussion. In Table I, the advances recorded in enzyme purification during the successive operations described above are noted. Purification procedure was accompanied by the analysis of preparations of enzyme

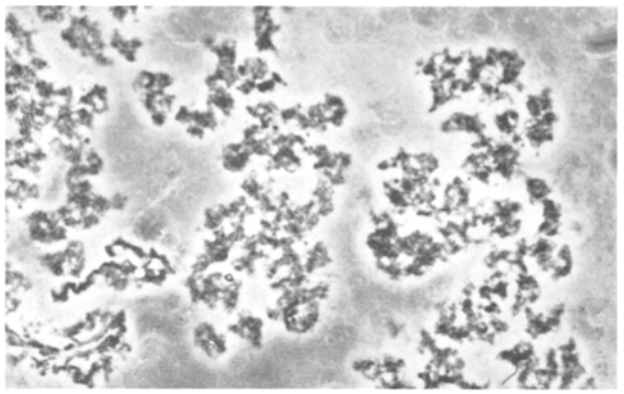
[1] D. H. CRUICKSHANK and F. A. ISHERWOOD, Biochem. J. *69*, 189 (1958).
[2] N. G. BAPTIST, Enzymologia *33*, 161 (1967).
[3] M. V. PATWARDHAN, Biochem. J. *75*, 401 (1960).
[4] D. D. DAVIES and R. J. ELLIS, Biochem. J. *78*, 623 (1961).
[5] O. H. LOWRY, N. J. ROSEBROUGH, L. A. FARR, R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).
[6] F. ROSEN, N. R. ROBERTS and C. A. NICHOL, J. biol. Chem. *234*, 476 (1953).
[7] A. TISELIUS, S. HJERTEN and O. LEVIN, Arch. Biochem. Biophys. *65*, 132 (1956).

Table I. Purification of L-alanine-2-oxoglutarate-aminotransferase extracted from *Glycine hispida* var. Cheepewa seeds. Substrate-DL-alanine

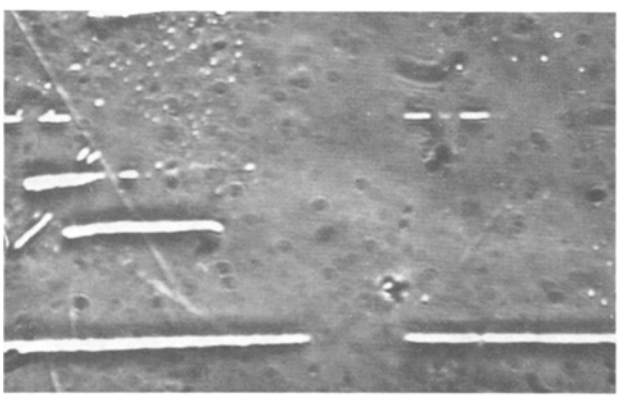
Fraction	Volume (ml)	Formed pyruvic acid (μM/min/37 °C)	Protein (mg/ml)	Protein in sample (mg)	Specificity (μM pyruvic acid/mg protein/ min/37 °C)	Purification factor	Yield (%)
Total extract	40	51,360	11.25	450	114.1	1.0	100
Fraction I from the column of Sephadex G-50 medium	50	47,050	5.15	275.5	183	1.6	91.4
Fraction IV from the column of DEAE cellulose SS	12	–	6.2	74.4	318.8	2.7	–
Fraction VI from the column of DEAE cellulose of the hydroxylapatite type	12	29,448	1.0	12	2,454	21.5	57.3
Fractions VII and VIII from the hydroxylapatite column	16	24,544	0.217	4.082	7,069	61.9	47.8
Fraction IX (or fractions X) from the column of Sephadex G-50 medium	2	1,543	0.029	0.058	26,448	231.9	3.0

purified by the method of disc electrophoresis on polyacrylamide gel[8].

Purified fractions (IX and (or) X, Table I) were employed for enzyme crystallization in the presence of 2-oxoglutaric acid with $(NH_4)_2SO_4$ (Figure 1, a) and ethyl alcohol (Figure 1, b). L-alanine: 2-oxoglutarate-aminotransferase crystals from $(NH_4)_2SO_4$ solution remind, as shape, of another transferase, isolated and crystallized by WADA and MORINO[9] from bovine liver.



a



b

Photomicrographs of samples of alanine: 2-oxoglutarate-aminotransferase isolated from *Glycine hyspida* var. Cheepewa. a) Crystallization from $(NH_4)_2SO_4$ solution. × 660. b) Crystallization from ethilic alcohol. × 660.

Table II. Action of certain cations upon the activity of vegetable L-alanine-2-oxoglutarate-aminotransferase

Nature of cation	Formed pyruvic acid ($\mu M \times 10^4$/min)	%
$AgNO_3$	221	46.3
$MgCl_2$	181	37.9
$MnCl_2$	383	80.3
$K_3Fe(CN)_6$	315	66.0
$CoCl_2$	238	49.9
$HgCl_2$	0.0	0.0
NaN_3	277	58.0
KCN	198	4.2
$ZnCl_2$	201	42.1
Control sample	477	100.00

Enzyme concentration in reaction medium 66.5 μg; pH 7.5; samples were incubated for 20 min at 37 °C, $1 \times 10^{-6} M$ cation solutions were used, while their final concentration in reaction medium was $1 M$.

Spectrum of absorption in ultraviolet, at different pH values, of L-alanine: oxoglutarate-aminotransferase preparation is not sensibly modified and tallies with MARTINEZ-CARRION and JENKIN's data[10] described for D-alanine: D-glutamate-transaminase isolated from bacterial cultures. The relationship A_{280}/A_{260} is in most cases between 1.18 and 1.27, very close to that of other transaminases isolated from plants[11] or of other vegetable transferases — 1.27[12].

Purified enzyme has an action pH optimum at 7.5 in potassium phosphate buffer and at 37 °C. At a temperature of 45 °C, after the first 10 min enzyme thermic denaturation probably begins, since, in this case, enzyme activity suddenly diminishes at exposal intervals of 20, 30 and 40 min.

Purified enzyme preparations manifest a high specificity transaminating DL-alanine at a high speed. Of other amino acids which can be submitted to transamination, attention is attracted only by serine, which is transaminated with α-oxoglutaric acid at a speed which forms about 59% of DL-alanine transamination intensity. L-leucine and L-phenyl alanine are not transaminated at a speed detectable by the method employed by us, while L-asparagine, L-valine and L-isoleucine are transaminated at a much lower speed. Similar results were likewise recorded by WILSON, KING and BURRIS for other vegetable transaminases[13].

Influence of different cations upon vegetable L-alanine: 2-oxoglutarate-aminotransferase activity is shown in Table II.

Michaelis constants, assessed by procedures described by DIXON and WEBB[14], were 31.2 mM for alanine, while for 2-oxoglutaric acid it is 0.936 mM.

At present new experiments are being undertaken concerning the connection between apoenzyme and phosphopyridoxal.

Zusammenfassung. L-Alanin-2 Oxoglutarataminotransferase aus Soya, *Glycine hispida*, wurde 230fach gereinigt und kristallisiert sowie einige physikalisch-chemische Eigenschaften (Michaelis Konstante, spezifische Wirkung, pH, Temperatureinfluss, ionale Wirkungen und Metaboliten) wurden studiert.

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[8] J. B. DAVIES, Ann. N.Y. Acad. Sci. *121*, 404 (1964).
[9] H. WADA and Y. MORINO, *Vitamins and Hormones. Advances in Research and Applications* (Eds. R. S. HARRIS, I. G. WOOL and J. A. LORAINE; Academic Press, New York – London 1964), vol. 22, p. 411.
[10] M. MARTINEZ-CARRION and T. W. JENKINS, J. biol. Chem. *240*, 3538 (1965).
[11] T. V. REDKINA, J. V. USPENSKAYA and V. L. KRETOVICH, Biokhimia *34*, 312 (1969).
[12] F. PETEK, E. WILLARROYA and J. E. COURTOIS, Europ. J. Biochem. *8*, 395 (1969).
[13] D. G. WILSON, K. W. KING and R. H. BURRIS, J. biol. Chem. *208*, 863 (1954).
[14] M. DIXON and E. C. WEBB, *The Enzymes* (Longmans Green and Co., London – New York, Toronto 1964).