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PROPERTIES OF D(+)-LYSOPINE DEHYDROGENASE FROM CROWN GALL TUMOUR TISSUE

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Summary

D(+)-Lysopine dehydrogenase of an octopine-type Crown Gall tumour has been partially purified and a number of kinetic parameters have been determined. D(+)-Lysopine dehydrogenase catalyzes the reductive condensation of pyruvate and one of at least six different L-amino acids, as well as the reverse reactions, with preferential use of NADP(H) as a cofactor. The optimal pH for both reductive and oxidative reactions has been determined. At pH 6.8, L-lysine has of all the amino acids the lowest K_m value, while at the same pH the highest V was found with L-arginine and L-histidine. The isoelectric point of D(+)-lysopine dehydrogenase is about 4.5.

Introduction

Since the discovery of a number of unusual amino acid derivatives in Crown Gall tumour tissue relatively little work has been carried out on the enzymes involved in the synthesis of these compounds. Till now, five amino acid derivatives have been identified, belonging to two different types of tumour; nopaline, exclusively found in nopaline tumours, and octopine, octopinic acid, lysopine and histopine, found in what can be called octopine tumours (Table I).

Morel and coworkers showed that the nature of the tumour depends on the infecting bacterial strain [6]. It was also shown that the bacteria can oxidize the amino acid derivatives formed in the tumour they cause, but not the amino acid derivatives belonging to the other tumour type [7]. Lejeune demonstrated that lysopine was formed by the reductive condensation of lysine and pyruvate (using the enzyme, D(+)-lysopine dehydrogenase as catalyst) in Crown Gall extracts [8]. Some additional information was obtained by Bomhoff with regard to the production of octopine [9]. More knowledge about the amino acid derivative enzymes could help to gain insight in the Crown Gall phenome-

non, especially as to the question of the genetic origin, bacterial or vegetative, and the metabolic function of these enzymes.

Materials and Methods

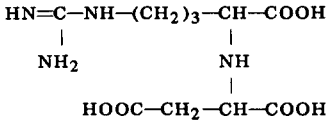
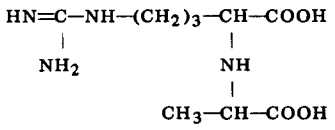
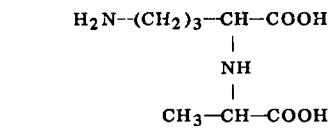
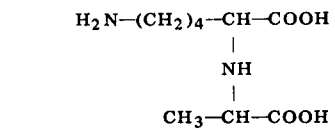
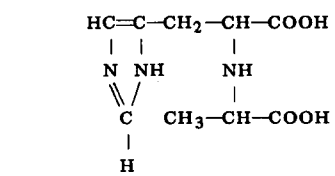
Chemicals. Bovine serum albumin: Koch Light Laboratories, ovalbumin: Nutritional Biochemicals Corporation, hydroxyapatite: Biorad, diethyl carbamic acid: Sigma, lactate dehydrogenase (EC 1.1.1.27): Boehringer. All substrates used were of analytical grade. D(+)-Lysopine was a generous gift of Dr. B. Lejeune, D-arginine and D-ornithine were a gift from Dr. L. Schattekerke.

Tumour tissue. Nicotiana tabacum var. White Burley leaf tumours were initiated by infection with *Agrobacterium tumefaciens* strain B6S3 and cultured in vitro on a hormone-free medium as described earlier [10].

Isolation procedure. All steps were carried out at 4°C. A typical isolation procedure started with 250 g fresh weight of tumour tissue. B6S3 tumour tissue was ground in a mortar with 1.25 ml per g fresh weight of 0.1 M ascorbic acid, 0.01 M diethyl carbamic acid and 0.1 M potassium phosphate (pH 6.3), which we refer to as buffer A. Cell debris was spun down at 12 000 × *g* for

TABLE I

AMINO ACID DERIVATIVES AS FOUND IN CROWN GALL TUMOURS

Chemical formula	name of compound (trivial name in square brackets)	Reference
	N^2-(1,3,-dicarboxypropyl)-L-arginine [nopaline]	1
	N^2-(D-1-carboxyethyl)-L-arginine [octopine]	2
	N^2-(D-1-carboxyethyl)-L-ornithine [oxtopinic acid]	3
	N^2-(D-1-carboxyethyl)-L-lysine [lysopine]	4
	N^2-(D-1-carboxyethyl)-L-histidine [histopine]	5

20 min. The pellet was re-extracted once with 0.5 ml of buffer A per g fresh weight. Both supernatant fractions were combined and 0.8 vols. of saturated $(NH_4)_2SO_4$ were added, while the pH was carefully adjusted to 6.3 with 0.5 M NaOH. After 30 min of gentle stirring the precipitate was pelleted down at 12 000 × *g* for 30 min. The pellet was discarded and to the supernatant was added 160 g/l of $(NH_4)_2SO_4$. The precipitate was pelleted down at 12 000 × *g* for 30 min and the pellet resuspended in 0.2 times the amount of supernatant volume of 0.02 M Tris · HCl (pH 7.5), 0.1 mM EDTA, 0.01 mM dithiothreitol, 1 mM L-arginine (buffer B). Dry hydroxyapatite was added to a concentration of 50 g/l and after 20 min of stirring eliminated by centrifugation at 12 000 × *g* for 10 min. The supernatant was diluted with two volumes of buffer B. DEAE-Sephadex (A50) equilibrated with buffer B, was added to a final concentration of 200 g/l (wet weight), as well as glycerol to a final concentration of 10% (v/v). After 15 min of gentle stirring the DEAE-Sephadex slurry was de-aerated, poured into a column (40 × 3 cm), allowed to settle and washed with three volumes of buffer B, containing 10% glycerol (v/v). Elution was carried out with 0.02 M Tris · HCl (pH 7.5), 0.1 mM EDTA, 0.01 mM dithiothreitol, 1 mM L-arginine, 10% glycerol (v/v), and 400 mM $(NH_4)_2SO_4$ (buffer C), at a rate of 0.2 ml/cm^2 per min. Absorbance at 280 nm, refractive index at 20° C ($n_D^{20°C}$) and enzyme activity of fractions were measured. Fractions showing enzyme activity were pooled and the enzyme was precipitated with 500 g/l of $(NH_4)_2SO_4$. The pellet was redissolved in 1/10 of the supernatant volume of buffer C and layered on a Sephadex G-100 column (30 × 3 cm), equilibrated with the same buffer. The column was eluted at a rate of 0.1 ml/cm^2 per min with buffer C. Absorbance was read at 280 nm and the enzyme activity of the fractions was measured. Fractions with enzyme activity, containing about 60% of the total activity eluted from the column, were pooled. The enzyme preparation thus obtained was stable for some days at 4° C and for many weeks at −20° C.

Enzyme assay. Enzyme activity was measured by following the reduction of NADP or the oxidation of NADPH at 340 nm with a Philips Unicam SP 1800, equipped with a temperature control device, at 30° C. Detection limit was 0.001 absorbance units, corresponding to 0.16 μM NADPH. One activity unit corresponds to the disappearance of 1 μmol of NADPH per min during octopine synthesis in a 0.1 M potassium phosphate buffer (pH 6.8), at 30° C, in the presence of 12 mM pyruvate, 16 mM L-arginine, and 0.06 mM NADPH. Test volume was 0.8 ml. The synthesis of octopine has been verified by electrophoresis and staining as described before [9].

Protein concentration. Protein concentration was determined with the Lowry method modified according to Bensadoun and Weinstein [11]. Bovine serum albumin was used as a standard.

Cellogel electrophoresis. Cellogel sheets were purchased from Chemetron, Milan. Runs were for 3 h at 4° C in 0.1 M sodium phosphate buffer (pH 6.8). Ovalbumin and bovine serum albumin were added as markers to a final concentration of 0.5 mg/ml each. Eight bands of 7 × 0.5 mm were applied on a 16 × 16 cm sheet, each band containing about 2 μl enzyme solution with a protein concentration of about 1.5 mg/ml. After the run one strip of Cellogel sheet was stained for 5 min in Amido Black (0.1% Amido Black in 45% metha-

nol/10% acetic acid/45% distilled water), followed by destaining for 30 min in 45% methanol/10% acetic acid/45% distilled water. The rest of the sheet was cut in strips (10 × 160 mm), each cut in segments of 2 × 10 mm. These segments were separately tested in 1 ml 0.1 M sodium phosphate (pH 6.8) 0.1 mM NADPH, 12 mM pyruvate and one of the following amino acids: 16 mM L-arginine, 32 mM L-histidine, 4 mM L-lysine, 30 mM L-glutamine, 10 mM L-methionine, 10 mM L-ornithine, or 16 mM L-alanine. After 120 min of incubation at 28°C absorbances were read at 340 nm. The method used here has been compared with the conventional method of dehydrogenase staining by tetrazolium salt formation. In our case, because of relatively low oxidation activity, this method was less adequate to obtain good pictures. Moreover, the expected reaction products, except for D(+)-octopine and D(+)-lysopine, were not available.

Results

Purification. Specific activities of D(+)-lysopine dehydrogenase at different stages of purification are given in Table II. The DEAE-Sephadex elution pattern and the Sephadex G-100 elution profile are given in Figs. 1 and 2 respectively. The final enzyme preparation contains about 0.1 mg/ml protein and has a specific activity of 1 unit per mg protein. This represents an 80-fold purification as compared to the crude extract. The enzyme yield is about 47%. All further determinations have been carried out with purified enzyme.

pH optima. Optimal pH values have been calculated for the reductive condensation of pyruvate and a number of L-amino acids. Results are given in Fig. 3. Optima for D(+)-octopine and D(+)-lysopine breakdown are shown in Fig. 4.

Michaelis constants. K_m and V values have been calculated for a number of substrates at pH 6.8 for the reductive reaction and at pH 8.4 for the oxidative reaction. Results are listed in Table III. Lineweaver-Burk plots for L-lysine, pyruvate and D(+)-lysopine are shown in Figs. 5, 6 and 7.

Assay of other substrates. Other substrates than those listed in Table III were assayed at the concentrations indicated. Results are summarized in Table IV.

TABLE II

PURIFICATION OF D(+)-LYSOPINE DEHYDROGENASE

Values have been calculated for 1 kg (fresh weight) of Crown Gall tumour tissue. Activities and protein content have been determined as described under Materials and Methods.

Purification step	mg protein	Activity (units · 10^3)	Specific activity (units/mg protein · 10^3)	Enzyme yield (%)
1. Crude extract	1320	16 700	13	100
2. 45—70% $(NH_4)_2SO_4$	648	17 400	27	104
3. Hydroxyapatite	372	15 200	41	91
4. DEAE-Sephadex	43.2	12 200	283	73
5. G-100 gel filtration	7.6	7 950	1045	47

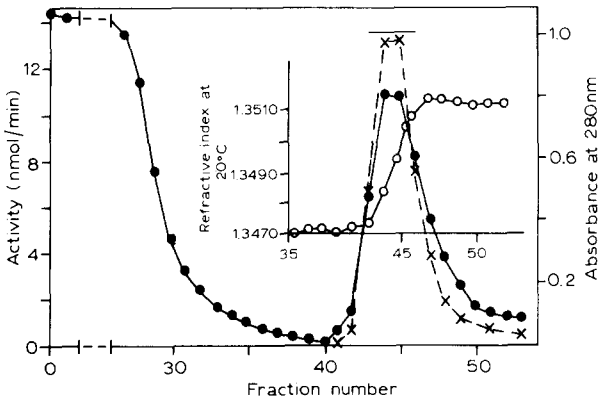


Fig. 1. DEAE-Sephadex chromatography. Elution pattern of D(+)-lysopine dehydrogenase. Technical details are given in the text. Fractions of 6 ml were collected, 0.1 ml per fraction was used to test enzyme activity. Fractions indicated by a bar were pooled. Enzyme activity (X———X), 280 nm (●———●), refractive index (○———○).

Identity of octopine cleavage products. D(+)-octopine breakdown was started in 0.5 mM NADP, 6 mM D(+)-octopine, and 0.1 M glycine (pH 9.6). When 130 nmol of NADP had been converted to NADPH, the reaction was stopped by denaturing the enzyme at 60°C for 15 min. After cooling to room temperature lactate dehydrogenase was added and its activity measured by following the disappearance of NADPH at 340 nm. As a control, a reaction mixture was immediately denatured, without prior incubation, and tested for the presence of pyruvate by measuring lactate dehydrogenase activity. No activity was found. In the incubated sample, at least 70% of the NADPH formed was re-oxidized, indicating that pyruvate is indeed one of the reaction products, L-arginine being the other one.

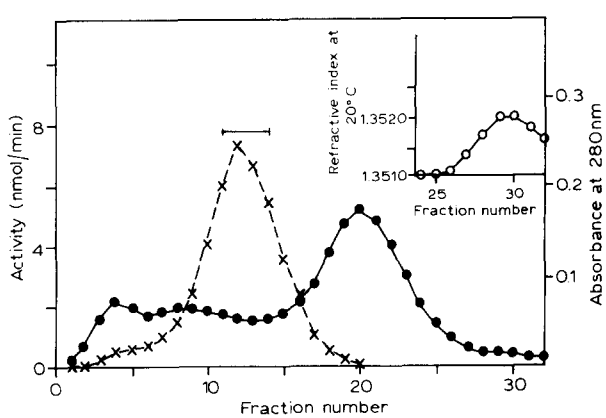


Fig. 2. Gel chromatography of D(+)-lysopine dehydrogenase on Sephadex G-100. Technical details are given in the text. Fractions of 6 ml were collected, 0.1 ml per fraction was tested for enzyme activity. Symbols as in Fig. 1.

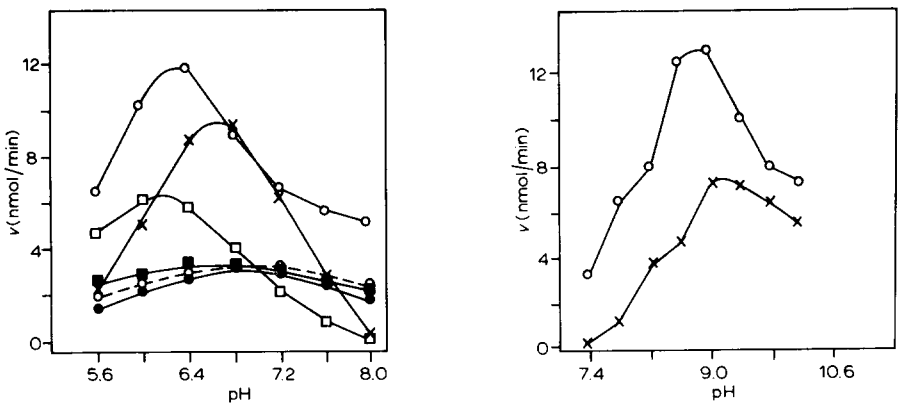


Fig. 3. pH-dependence of reductive condensation reactions catalyzed by D(+)-lysopine dehydrogenase. Reactions were carried out in citrate/phosphate buffer, 0.1 M, 12 mM pyruvate, 0.125 mM NADPH, and one of the following amino acids: 4 mM L-lysine (●———●), 10 mM L-methionine (○———○), 10 mM L-ornithine (□———□), 16 mM L-arginine (X———X), 32 mM L-histidine (○———○), 30 mM L-glutamine (■———■). Temperature: 30°C.

Fig. 4. pH-dependence of oxidation reactions catalyzed by D(+)-lysopine dehydrogenase. Reaction mixtures: 0.1 M, Tris · HCl, 0.5 mM NADP, 0.8 mM D(+)-lysopine (○———○), or 4 mM D(+)-octopine (X———X).

TABLE III

K_m AND V VALUES OF D(+)-LYSOPINE DEHYDROGENASE FOR DIFFERENT SUBSTRATES

Reaction temperature: 30°C. The reactions were linear for at least 15 min.

Synthesis	K_m (mM)	V (in % $V_{L\text{-lysine}}$)
(12 mM pyruvate, 0.125 mM NADPH, 0.1 M sodium phosphate, pH 6.8)		
L-lysine	0.56	100
L-methionine	1.3	125
L-ornithine	2.3	128
L-arginine	4.4	264
L-histidine	6.1	255
L-glutamine	6.1	110
L-citrulline	17	240
(4 mM L-lysine, 0.125 mM NADPH, 0.1 M sodium phosphate, pH 6.8)		
Pyruvate	0.23	
(4 mM L-lysine, 12 mM pyruvate, 0.1 M sodium phosphate, pH 6.8)		
NADPH	$3 \cdot 10^{-3}$	
Breakdown	**K_m (mM)**	**V (in % $V_{L\text{-lysine}}$)**
(0.5 mM NADP, 0.1 M Tris · HCl, pH 8.4)		
D(+)-lysopine	0.16	29
D(+)-octopine	0.8	18
(4 mM D(+)-lysopine, 0.1 M Tris · HCl, pH 8.4)		
NADP	$10 \cdot 10^{-3}$	

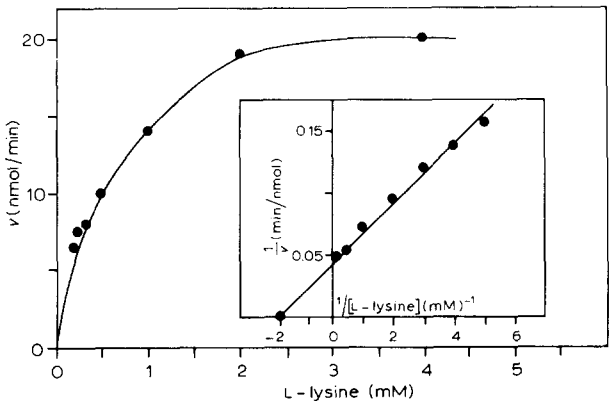


Fig. 5. K_m determination of L-lysine for D(+)-lysopine dehydrogenase. Reaction mixture: 0.1 M sodium phosphate (pH 6.8), 12 mM pyruvate, 0.125 mM NADPH, varying concentrations of L-lysine. Temperature: 30°C. Inset: Lineweaver-Burk plot of same determination.

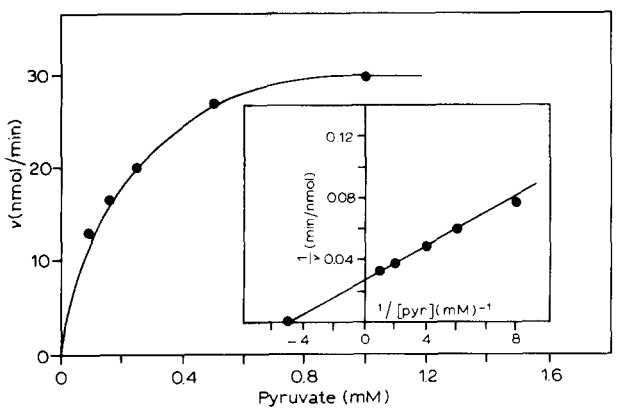


Fig. 6. K_m determination of pyruvate for D(+)-lysopine dehydrogenase. Reaction mixture: 0.1 M sodium phosphate (pH 6.8), 4 mM L-lysine, 0.125 mM NADPH, varying concentrations of pyruvate. Temperature: 30°C. Inset: Lineweaver-Burk plot of same determination.

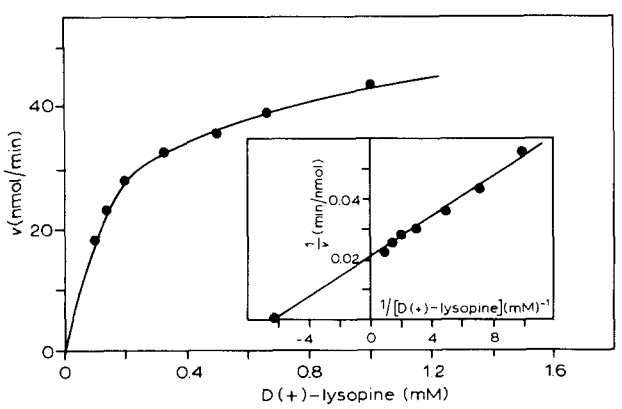


Fig. 7. K_m determination of D(+)-lysopine for D(+)-lysopine dehydrogenase. Reaction mixture: 0.1 M Tris · HCl (pH 8.4), 0.5 mM NADP, varying concentrations of D(+)-lysopine. Temperature: 30°C. Inset: Lineweaver-Burk plot of same determination.

TABLE IV

ACTIVITY OF D(+)-LYSOPINE DEHYDROGENASE WITH DIFFERENT SUBSTRATES

Reaction temperature: 30°C. The reactions were linear for at least 15 min. Activity is expressed in % of activity obtained with the same material in the presence of 16 mM L-arginine, 12 mM pyruvate, 0.125 mM NADPH, 0.1 M sodium phosphate, pH 6.8. Detection limit: 1%

Substrate	Concentration (mM)	Activity
(12 mM pyruvate, 0.125 mM NADPH, 0.1 M sodium phosphate, pH 6.8)		
L-arginine	16	100
D-arginine, D-ornithine	16	4
D-histidine	25	0
L-leucine	5	4
L-cysteine	5	5
L-aspartic acid, L-valine, L-proline, L-threonine, L-isoleucine, L-tryptophan L-glutamic acid, L-asparagine,	5	0
L-phenylalanine	9	0
L-alanine, glycine	16	0
L-tyrosine	2	0
L-methionyl-L-alanine	5	0
Lysozyme (with terminal lysine)	2	0
(16 mM L-arginine, 0.125 mM NADPH, 0.1 M sodium phosphate, pH 6.8)		
α-Ketoglutaric acid	12	0
α-Ketobutyric acid	12	24
Glyoxylic acid	12	12
(16 mM L-arginine, 12 mM pyruvate, 0.1 M sodium phosphate, pH 6.8)		
NADH	0.125	20

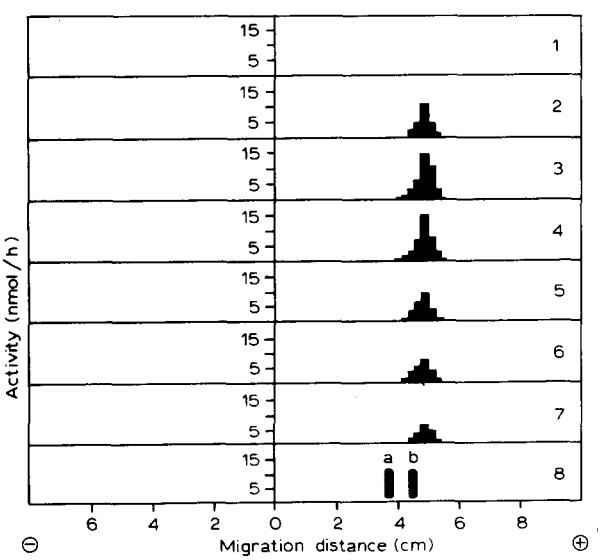


Fig. 8. Cellogel electrophoresis of D(+)-lysopine dehydrogenase. Technical details are given in the text. 1, L-alanine (16 mM); 2, L-methionine (10 mM); 3, L-histidine (32 mM); 4, L-arginine (16 mM); 5, L-ornithine (10 mM); 6, L-glutamine (30 mM); 7, L-lysine (4 mM); 8, reference proteins: a, bovine serum albumin (isoelectric point: 4.9); b, ovalbumin (isoelectric point: 4.6).

Cellogel electrophoresis. The Cellogel electrophoresis data are summarized in Fig. 8. They suggest, that D(+)-lysopine dehydrogenase is one enzyme, catalyzing the reductive condensation of pyruvate with at least six different L-amino acids: L-arginine, L-histidine, L-lysine, L-glutamine, L-methionine and L-ornithine. All activities are located at the same distance from the application point. The same result was obtained for D(+)-lysopine and D(+)-octopine by the tetrazolium salt staining method.

Discussion

Although a certain amount of information with regard to the synthesis of lysopine has been obtained by Lejeune [8], and some additional information on octopine synthesis by Bomhoff [9], their studies were hampered by the lack of stability of their preparations. The present isolation method makes it possible to obtain the D(+)-lysopine dehydrogenase of octopine tumours in a stable form, much more purified and free of interfering NADPH-oxidizing activities. Stabilization of the enzyme before the Sephadex G-100 filtration step could be achieved by the combined use of $(NH_4)_2SO_4$ and glycerol. After Sephadex G-100 fractionation the enzyme is also stable in a buffer without $(NH_4)_2SO_4$. The Sephadex G-100 profile of D(+)-lysopine dehydrogenase agrees with the molecular weight estimate of 35 000 reported by Lejeune [8]. In contrast to earlier findings, the enzyme prefers NADP(H) to NAD(H). This makes it different from the analogous enzyme D(+)-octopine dehydrogenase (EC 1.5.1.11) in animals [12]. Since D-(+)-lysopine dehydrogenase does not accept D-amino acids, we conclude that the enzyme is stereospecific with respect to the amino acid substrate. L-lysine, L-arginine, L-ornithine and L-histidine, as could be expected from the amino acid derivatives found in Crown Gall tissues, are good substrates. However, L-methionine, L-glutamine and L-citrulline are also accepted. The probable products are N^2-(D-1-carboxyethyl)-L-methionine, N^2-(D-1-carboxyethyl)-L-glutamine and N^2-(D-1-carboxyethyl)-L-citrulline respectively. Kemp recently reported the occurrence of histopine in Crown Gall octopine tumours [5]. Our results suggest, that other condensation products might be found, especially the methionine derivative, since the enzyme shows considerable affinity towards L-methionine. This high affinity shows also, that basic amino acids are not the only possible substrates as has been suggested [5]. The enzyme displays a broad specificity towards α-keto acids, since α-ketobutyric acid and glyoxylic acid can both form condensation products with L-arginine. However, α-ketoglutaric acid cannot be used, which excludes the production of nopaline. We propose that the enzyme should be called D(+)-lysopine dehydrogenase rather than D(+)-octopine dehydrogenase, since the enzyme shows the highest affinity towards L-lysine, while at the same time, D(+)-lysopine is better accepted than D(+)-octopine. D(+)-lysopine dehydrogenase does not accept the N-terminal lysine of lysozyme, nor the dipeptide L-methionyl-L-alanine, suggesting that the enzyme is not able to modify the N-terminal amino acids of protein molecules. Experiments are in progress to characterize also the nopaline-synthetizing enzyme(s) of nopaline tumours. Preliminary data have shown this enzyme system to be quite different D(+)-lysopine dehydrogenase, since it has a much higher molecular weight, and other stability characteristics (Otten, L.A.B.M., unpublished).

References

1 Goldmann, A., Thomas, D.W. and Morel, G. (1969) C.R. Acad. Sc. Paris 268, 852—854
2 Lioret, C. (1956) Bull. Soc. Fr. Physiol. Vég. 2, 76
3 Ménagé, A. and Morel, G. (1964) C.R. Acad. Sc. Paris 259, 4795—4796
4 Ménagé, A. and Morel, G. (1965) 261, 2001—2002
5 Kemp, J.D. (1977) Biochem. Biophys. Res. Commun. 74, 862—868
6 Goldmann, A., Tempé, J. and Morel, G. (1968) C.R. Soc. Biol. 162, 630—631
7 Petit, A., Delhaye, S., Tempé, J. and Morel, G. (1970) Physiol. Vég. 8, 205—213
8 Lejeune, B. (1973) Ph.D. Thesis, Paris
9 Bomhoff, G.H. (1974) Ph.D. Thesis, Leiden
10 Dons, J.J.M. (1975) Ph.D. Thesis, Leiden
11 Bensadoun, A. and Wettstein, D. (1976) Anal. Biochem. 70, 241—250
12 Thoai, N.V., Huc, C., Pho, D.B. and Olomucki, A. (1969) Biochim. Biophys. Acta, 191, 46—57