SOME PHYSICO-CHEMICAL PROPERTIES OF L-PHENYLALANINE AMMONIA-LYASE OF WHEAT SEEDLINGS

J. NARI, Ch. MOUTTET, M.H. PINNA and J. RICARD
Laboratoire de Physiologie Cellulaire Végétale associé au C.N.R.S., Université d'Aix-Marseille, Centre de Luminy, Marseille, France

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1. Introduction

L-phenylalanine ammonia-lyases* are enzymes which convert L-phenylalanine to *trans*-cinnamate. They are widely distributed in higher plants [1] and seem to fall into two groups. The enzymes from monocotyledons probably have, in addition, a tyrosine ammonia-lyase activity, whereas those of dicotyledons do not [2]. The purest L-phenylalanine ammonia-lyase preparation so far obtained, was extracted from potato tuber [3, 4], and has been estimated [3] to be about 80% homogeneous as defined by various criteria (molecular sieving, sucrose density gradient centrifugation, 4% polyacrylamide gel electrophoresis). The only known physico-chemical characteristics of this enzyme are the values of the Stoke's radius, of the sedimentation constant and of the molecular weight (estimated by sucrose density gradient centrifugation and molecular sieving [3, 5]). To the best of our knowledge, nothing has been published as to the homogeneity of the enzyme preparations obtained from monocotyledons [2, 5].

It is thus the main goal of this work to determine some physico-chemical characteristics of the homogeneous enzyme purified from a monocotyledonous plant (wheat seedlings).

2. Methods

The enzyme activity is defined following Zucker [6]. Protein concentration is estimated by the technique of Lowry et al. [7]. Polyacrylamide gel electrophoresis (either 7.5% or 4% gels) is carried out by the method of Davis [8]. Protein bands are stained with Amido black or detected by spectrophotometry using the Gilford 2410 device. Enzyme activity in the gels is measured in the presence of L-phenylalanine with the same apparatus, by following the appearance of *trans*-cinnamate at 290 nm. Polypeptide chain determination is effected by the method of Shapiro et al. [9] as modified by Weber and Osborn [10]. Amino acid analyses are performed with a Beckman Unichrom analyzer following the technique of Moore et al. [11, 12]. Representative Schlieren patterns are obtained with a Spinco model E analytical centrifuge. Centrifugations are performed at 50,740 rpm (bar angle 50°) in a 0.1 M borate buffer, pH 8.7. The value of the sedimentation constant $S^0_{20,w} \times 10^{13}$ is obtained by extrapolating at infinite dilution the values of the experimentally obtained sedimentation constants $S^c_{20,w} \times 10^{13}$. The Stoke's radius of the protein is estimated by analytical molecular sieving [13] through Sephadex G-200 (1.6 × 110 cm). Molecular weight of the pure enzyme is estimated by analytical centrifugation [14] and molecular sieving, as well as from the amino acid composition [15].

3. Results

The enzyme is isolated following a method derived from that of Havir and Hanson [4]. The acetone powder obtained from wheat seedlings (coleoptile, leaf and roots) is extracted with a 0.1 M borate buffer

* L-phenylalanine ammonia-lyase: EC 4.3.1.5.

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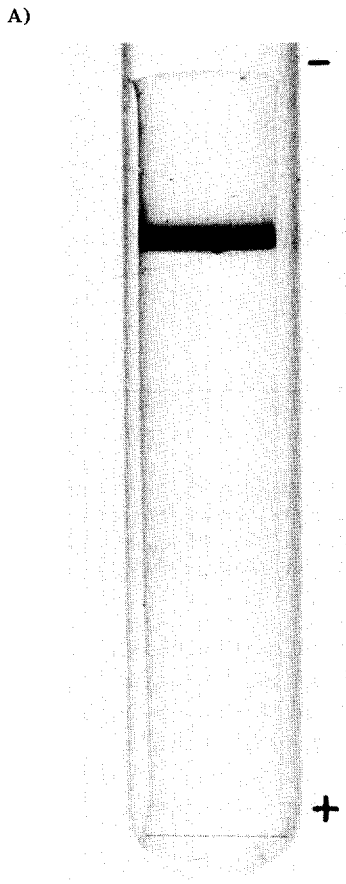

Fig. 1. Polyacrylamide gel electrophoresis of wheat L-phenylalanine ammonia-lyase. A) L-phenylalanine ammonia-lyase (100 μg) is subjected to electrophoresis (7.5% gel) and stained with Amido black.

pH 8.7*. The filtrate is acidified to pH 5.6 and fractionated with protamine sulfate (10% of the protein concentration of the extract). The supernatant is again fractionated with ammonium sulfate at 60% saturation. The precipitate, resuspended in a 0.1 M borate buffer pH 8.7, is submitted to molecular sieve chromatography on Agarose columns (Bio-Gel A-1.5 M, 200–400 mesh, 4 X 40 cm). The active fractions are collected and chromatographed on

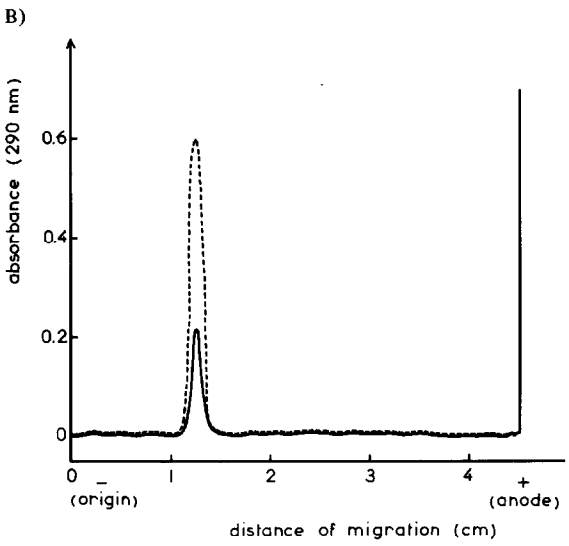

Fig. 1. Polyacrylamide gel electrophoresis of wheat L-phenylalanine ammonia-lyase. B). Electrophoretic pattern of L-phenylalanine ammonia-lyase. The amount of enzyme subjected to electrophoresis (7.5% gel) is 60 μg. The full line is the spectrophotometric trace (Gilford 2400 S spectrophotometer with the 2410 gel device) at 290 nm, corresponding to the absorption of the protein. The dotted line is the spectrophotometric trace in the presence of 0.1 M L-phenylalanine (after 10 min). At 290 nm, L-phenylalanine does not absorb whereas *trans*-cinnamate does.

DEAE-cellulose columns (DE 32 Whatman) equilibrated in a 0.05 M phosphate buffer pH 6.8. The enzyme is eluted with a linear gradient of potassium chloride. The active fractions are concentrated in a 0.005 M phosphate buffer, by ultrafiltration on Diaflo membranes (Amicon), then submitted to a hydroxyapatite chromatography. Elution of the column (2.4 X 5 cm Bio-Gel HTP) is effected by stepwise addition of phosphate buffer (from 0.005 M up to 0.1 M). The active fractions are again concentrated in a 0.1 M borate buffer pH 8.7 on a Diaflo membrane.

The phenylalanine ammonia-lyase thus obtained has a specific activity of about 800 mU/mg of protein†. The enzyme preparation is homogeneous on polyacrylamide gel electrophoresis (fig. 1) even at

* No difference is observed in the properties of the enzyme extracted from roots and from coleoptiles. Moreover, no light induction of phenylalanine ammonia-lyase is observed in the seedlings.

† In fact, this specific activity is underestimated, because of the partial denaturation of the enzyme during its purification. The true specific activity must be close to 1200 mU/mg of protein.

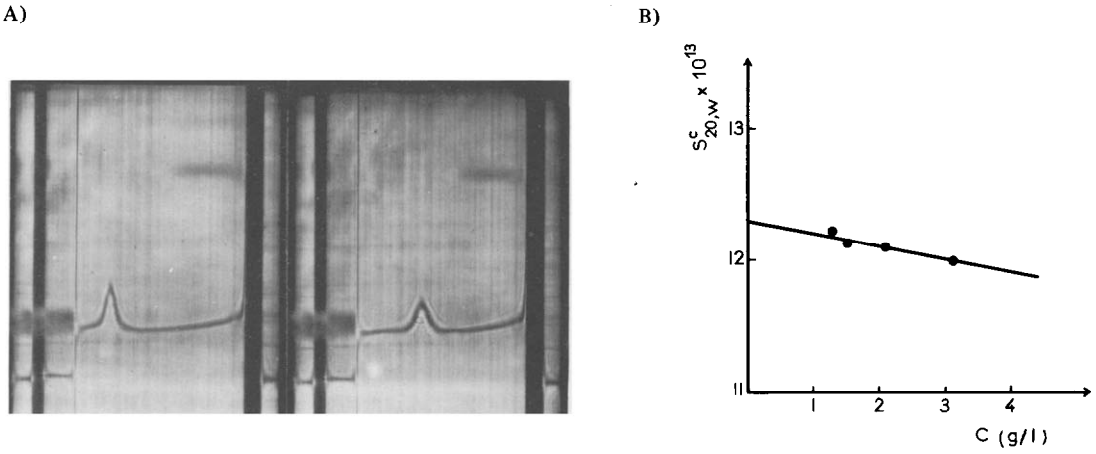

Fig. 2. Ultracentrifugation of wheat L-phenylalanine ammonia-lyase. A) Ultracentrifugation diagrams of the L-phenylalanine ammonia-lyase. The enzyme concentration is 2.05 mg/ml (0.1 M borate buffer, pH 8.7). Migration is from left to right (rotation speed 50,740 rpm, bar angle 50°, temperature 20°). Pictures are taken after 16 min (left) and 32 min (right). B) Estimation of the sedimentation constant of L-phenylalanine ammonia-lyase at infinite dilution. The measurements are made under conditions identical to those of fig. 2A.

high protein concentration. The protein peak and the activity peak coincide (fig. 1). It should be noted that some protein contaminations are not detected on the 4% gels used by Havir and Hanson [3], whereas these contaminations are clearly seen on 7.5% gels. The enzyme preparation is still homogeneous on analytical centrifugation (fig. 2). Its sedimentation constant, estimated at infinite dilution of the protein, is 12.3 (fig. 2). The molecular weight of the pure enzyme, obtained on analytical centrifugation, is 325×10^3 daltons. Analytical molecular sieve chromatography on Sephadex G-200 gives a very similar value (320×10^3 daltons). The Stoke's radius of the enzyme, estimated by this method, is 63 Å.

The amino acid composition of the pure protein is given in table 1. The enzyme molecule contains about 2800 amino acid residues and is particularly rich in dicarboxylic amino acids (aspartic and glutamic acids). The above composition allows a new estimation of the molecular weight [15]. The value obtained (329×10^3 daltons) is very close to the previous ones. The homogeneous phenylalanine ammonia-lyase preparation has no phenolase activity but still possesses a tyrosine ammonia-lyase activity.

Electrophoresis of the pure enzyme in denaturating conditions [9, 10] shows the existence (fig. 3) of two different polypeptide chains (called α and β), with molecular weights of 75×10^3 daltons and 85×10^3 daltons, respectively. Because of the estimated value of 320×10^3 daltons for the molecular weight of the whole enzyme, the above results suggest the existence of two α chains (150×10^3 daltons) and two β chains (170×10^3 daltons) in the molecule of phenylalanine ammonia-lyase.

4. Discussion

The purification procedure described in the present report exhibits many similarities with the original method of Havir and Hanson [3, 4]. However, the slight modifications we have introduced (use of hydroxyapatite chromatography with stepwise elution of the column, concentration of the extract with Diaflo membranes . . .) are probably sufficient to explain the high degree of purity of the enzyme preparation as shown by the fact that no visible contamination can be detected on polyacrylamide gel electrophoresis and analytical centrifugation.

Since the homogeneous phenylalanine ammonia-lyase preparation still exhibits tyrosine ammonia-lyase activity, it is highly probable that the pure enzyme possesses both activities. This conclusion is consistent with previous results [2].

In contrast with the situation previously described for dicotyledonous plants [3, 16], no isoenzymes

Table 1
Amino acid composition of wheat L-phenylalanine ammonia-lyase.

	A	B
Lysine	5.93	148
Histidine	2.59	61
Arginine	5.65	118
Asp-X[a]	10.62	290
Threonine	4.95	151
Serine	5.70	197
Glu-X[b]	14.08	294
Proline	4.99	158
Glycine	4.97	241
Alanine	7.17	293
½ Cystine[c]	1.70	51
Valine	6.83	212
Methionine	2.35	57
Isoleucine	5.60	156
Leucine	8.31	230
Tyrosine	3.51	70
Phenylalanine	4.97	109
Total number of residues		2836

A. Percentages (g of residues per 100 g protein ± 2%).
B. Moles per mole protein.
a Aspartic acid plus asparagine.
b Glutamic acid plus glutamine.
c Determined after performic oxidation (time of hydrolysis 20 hr).

Hydrolysis is run during 20 hr and 70 hr, and duplicate samples are used for each time. The values for serine, threonine and methionine are extrapolated to zero time of hydrolysis, those for valine, isoleucine and leucine are determined after 70 hr hydrolysis. For the other residues (with the exception of cysteine too), each value is the mean of 4 experimental results (2 after 20 hr hydrolysis, the 2 others after 70 hr).

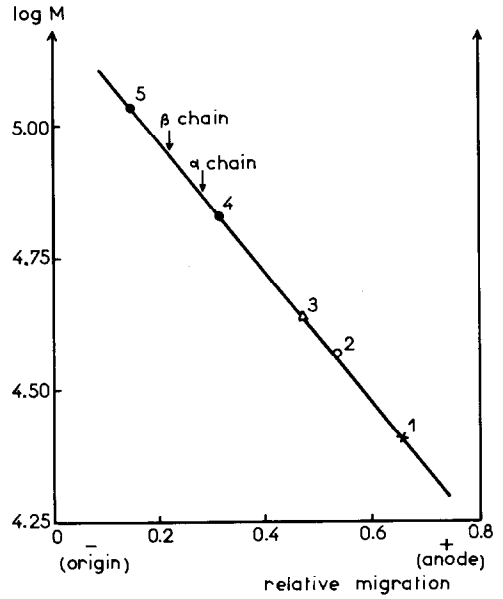

Fig. 3. Determination of the molecular weights of α and β polypeptide chains of L-phenylalanine ammonia-lyase. Gels (5%) are prepared by the method of Shapiro et al. [9]. 15 μg of each standard protein are denatured at pH 7 (8 M urea, 1% sodium dodecylsulfate and 1% β-mercaptoethanol). The logarithm of the molecular weight is plotted against the relative distance of migration.
1 (x): α-Chymotrypsinogen (25.7 × 10^3 daltons);
2 (○): Pepsin (35 × 10^3 daltons);
3 (△): Ovalbumin (43 × 10^3 daltons);
4 (●): BSA (68 × 10^3 daltons);
5 (●): BSA dimer (136 × daltons).
Each point is the mean of eight experiments.

have been found in the wheat seedlings. Moreover, there was no observable light effect on the enzyme level within the plant.

The molecular weight, the sedimentation constant, and the Stoke's radius of the homogeneous enzyme have values very close to those previously obtained for different enzyme preparations [3, 5].

The existence of four polypeptide chains in the phenylalanine ammonia-lyase molecule, raises the important question of subunit number. This point, along with the cooperativity of the enzyme, will be investigated at length in a forthcoming paper.

Acknowledgements

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