Phytochemistry, Vol. 29, No. 8, pp. 2411–2414, 1990.
Printed in Great Britain.

0031–9422/90 $3.00+0.00

CHARACTERIZATION OF MAIZE POLYAMINE OXIDASE

RODOLFO FEDERICO, ALESSANDRA CONA, RICCARDO ANGELINI, M. EUGENIA SCHININÀ* and ANNA GIARTOSIO*

Dipartimento di Biologia Vegetale; *Dipartimento di Scienze Biochimiche, Università "La Sapienza", P. le Aldo Moro 5, 00185 Rome, Italy

(*Received* 2 *January* 1990)

Key Word Index—*Zea mays*; Gramineae; maize; cell walls; polyamine oxidase.

Abstract—Some structural and biochemical characteristics of polyamine oxidase (PAO) purified from maize shoots have been examined. The enzyme has only alanine as *N*-terminal amino acid and its *N*-terminal sequence shows a significant degree of homology with tryptophan 2-monooxygenase from *Pseudomonas syringae* pv. *savastanoi*. The pH optimum for the stability of the native enzyme is 5, similar to that of the barley leaf enzyme. Calorimetric analysis shows a single two-state transition at pH 6 with T_m 49.8°. At pH 5 the thermal stability is increased by more than 14°. Amine oxidation products, Δ^1-pyrroline and diazabicyclononane, are competitive inhibitors of PAO activity (apparent $K_i = 400$ and 100 μM respectively). Moreover these compounds improve the thermal stability of the enzyme. N^1-Acetylspermine, which is a good substrate for mammalian PAO, acts as a non-competitive inhibitor for the plant enzyme.

INTRODUCTION

Plant polyamine oxidases (PAOs, EC 1.4.3.4) are flavin nucleotide-containing enzymes involved in the terminal catabolism of polyamines. These enzymes have been found only in the monocots [1] and they have been characterized in barley [2], maize [3, 4], millet [5], water hyacinth [6] and oat [7, 8]. Mammalian PAO, preferentially catalyses the oxidative cleavage of N^1-acetylpolyamines to form putrescine and spermidine plus monoacetylpropionaldehyde [9]. The plant enzymes are specific for spermidine and spermine, which are oxidatively deaminated to 4-aminobutyraldehyde and 1-(3-aminopropyl)-4-aminobutyraldehyde, present in solution mainly as Δ^1-pyrroline and diazabicyclononane, respectively [1]. These enzymes are quite stable on storage, have the highest stability at acidic pH and lose activity during oxidative reaction [10, 11]. The association of PAO with cell walls [12, 13] suggests that the enzyme could have some specific roles in the apoplast, such as the control of apoplastic polyamine level [14] and the production of H_2O_2 essential for peroxidase activity [13]. The latter enzymes are involved in crucial events of wall development such as cross-link formation, lignification and suberization [15–17]. Moreover the products of polyamine oxidation can be effective in the prevention of attacks by pathogens [18]. The present paper describes some molecular properties of maize PAO

RESULTS AND DISCUSSION

N-Terminal amino acid sequence of maize polyamine oxidase

Polyamine oxidase was purified from cell walls of *Z. mays* by a two-step procedure [4], whose salient features are the extraction of the enzyme from the washed cell-walls with high ionic-strength solution, CM-cellulose, and hydroxylapatite chromatography. Disc gel electrophoresis, either in absence or in presence of sodium dodecyl sulphate (SDS), shows that the enzyme is homogeneous. The *N*-terminal amino acid sequence of maize PAO is as follows:

H_2N-Ala-Thr-Val-Gly-Pro-Arg-Val-Ile-Val-Val-Gly-Ala-Gly-Met-Ser-Gly-Ile-Ser-Ala-Ala-Lys-Arg-Leu-Ser-Glu-Ala-Gly-Ile-Thr-Asp-Leu-Leu-Ile-. . . .

Alanine is the only *N*-terminal amino acid observed. The *N*-terminal sequence of maize PAO could be aligned with residues 35 to 67 (sequence translated from the DNA sequence) of tryptophan 2-monooxygenase from *Pseudomonas syringae* pv. *savastanoi* [19]. Per cent identity was 48.5, per cent similarity was 72.7.

Inhibition of enzyme activity by reaction products

Because a loss of enzymatic activity during polyamine degradation was observed for barley [20] and oat enzyme [10], we tested the effects of the reaction products on PAO activity. 1-(3-aminopropyl)-4-Aminobutyraldehyde and 4-aminobutyraldehyde were prepared as described in experimental. Both compounds act as reversible competitive inhibitors with apparent K_i of 100 and 400 μM, respectively, as determined by analysis of Lineweaver–Burk plots [21]. 1,3-Diaminopropane (1 mM) and H_2O_2 (1 mM) did not greatly affect the enzyme activity. These results suggest that the observed loss of PAO activity during the oxidation of spermine and spermidine is not due to H_2O_2 or 1,3-diaminopropane, but to the aminoaldehydes produced during the reaction. Moreover the ability to reversibly inhibit PAO activity could represent a control mechanism at the H_2O_2 and

aminoaldehyde level, which are potential toxic compounds. N^1-Acetylspermine, which is oxidatively cleaved by mammalian PAO to form spermidine [9], acts as a non-competitive inhibitor ($K_i = 4\ \mu M$) for the maize enzyme, as also observed for the oat enzyme [8].

Calorimetric analysis and thermal stability

The stability of the maize enzyme was determined over a range of pH values from 3.5 to 7, either by heating the protein at 55 and 64° for 30 min and measuring the residual activity, or by following the loss of enzyme activity at 55° as a function of time. The enzyme, which has an optimum pH for the activity of *ca* 5, is very stable upon heating in the range of pH 4–5.5 (Fig. 1A). Conversely at pH > 7 a rapid and irreversible loss of activity can be observed even at room temperature. Diazabicyclononane and Δ^1-pyrroline, at a concentration inhibiting 60% of the enzymatic activity, greatly improve the thermal stability of PAO, suggesting a strong interaction between aminoaldehydes and the enzyme (Fig. 1B). In the calorimetric scan of PAO at pH 6 a single endothermic peak is noticed (Fig. 2A). A small exotherm at 65.6° is probably due to aggregation of the denatured material as the recovered solution is turbid. No heat effect is observed in a subsequent scan of the heated material. Deconvolution of the trace shows that the curve is well-described by a two-state transition characterized by T_m (temperature of maximum heat capacity) 49.8°, ΔH_c (calorimetric denaturation enthalpy change) 182 kcal mol^{-1}, ΔH_{vh} (Van't Hoff denaturation enthalpy change) 182 kcal mol^{-1}, ΔC_p (heat capacity change upon denaturation) 6.3 kcal mol^{-1} (Fig. 2B). Denaturation appears therefore to be a single cooperative event involving the whole molecule [22]. The noticeable increase of C_p upon denaturation suggests a high degree of hydrophobic interactions in the native protein [23]. On heating in the presence of a great excess (5 mM) of spermidine no endotherm is noticed up to 85°, but a large exotherm at 77° is present (Fig. 2C). The increase of thermal stability is, in this case, obscured by the precipitation process. These phenomena could be ascribed to the Δ^1-pyrroline produced during the reaction. Actually, as described above, this compound improves the thermal stability of the enzyme. At pH 5, in accord with the activity measurements, the protein is more stable; the denaturation process, which is again partially obscured by precipitation, occurs with a $T_m \geq 63°$ (Fig. 2D). The present results, beside helping the chemical and catalytic characterization of PAO, suggest that the enzyme has a considerable stability in the physiological pH range of the apoplast.

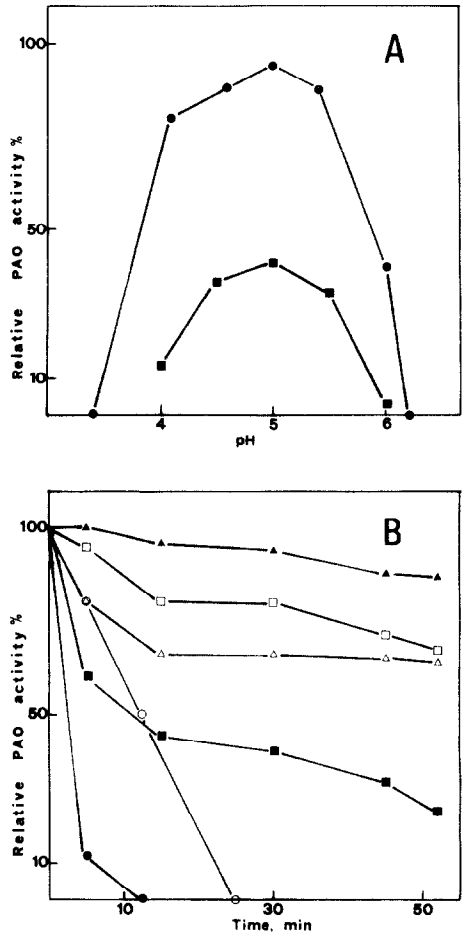

Fig. 1. Thermal stability of maize PAO determined as a function of time, pH and aldehyde incubation. (A) Purified maize PAO was incubated at 55° (●) and 64° (■), in a 3.5–7 range of pH units and the residual enzyme activity was determined after 30 and 15 min, respectively; (B) purified maize PAO was incubated at 55° at different pH values; and residual enzyme activity was determined over a 50 min period; the effect of Δ^1-pyrroline on enzyme thermal stability was determined by incubation of PAO with 10 mM Δ^1-pyrroline at pH 6. (▲) pH 5; (□) pH 4.1; (△) 10 mM Δ^1-pyrroline, pH 6; (■) pH 6; (○) pH 3.4; (●) pH 6.6. PAO activity is expressed as per cent of the initial value for each pH.

EXPERIMENTAL

Chemicals. N^1-Acetylspermine, spermine tetrahydrochloride, spermidine trihydrochloride and 1,3-diaminopropane were from Sigma; 4-aminobutyraldehyde diethylacetal was obtained from Aldrich; hydroxylapatite and carboxymethyl cellulose were from Bio-Rad. All other chemicals were obtained as pure commercial products.

Enzyme purification and assay. PAO was purified as previously described [4] from shoots of 10-day-old *Z. mays* L. seedlings grown at 25° in a greenhouse. Enzymatic activity was estimated at 37° using a Hansatech oxigraph equipped with a Clark electrode, according to ref. [24]. SDS gel electrophoresis was performed according to the method of ref. [24]. Native electrophoresis was carried out according to ref. [6].

Δ^1-Pyrroline and diazabicyclononane were prepared by incubation of spermidine or spermine (0.01 mol), respectively, with 2 μKat PAO and 2 μKat catalase, both bound to hydroxylapatite in 10 ml 0.05 M K-Pi buffer, pH 5. Incubation was performed for 10 min at 37° under an air flux. The reaction was terminated, separating the enzyme bound to hydroxylapatite from the reaction products by centrifugation at 500 g for 5 min. The supernatant was applied to a column of Dowex-50 (H^+ form) ion exchange resin and the aminoaldehydes were eluted with 2 M HCl. The pH of the fractions containing aldehydes (determined according to ref. [25]) was raised to 6 with 2 M KOH immediately before use. After use the hydroxylapatite was washed × 3 with 0.05 M K-Pi, pH 5, and stored at

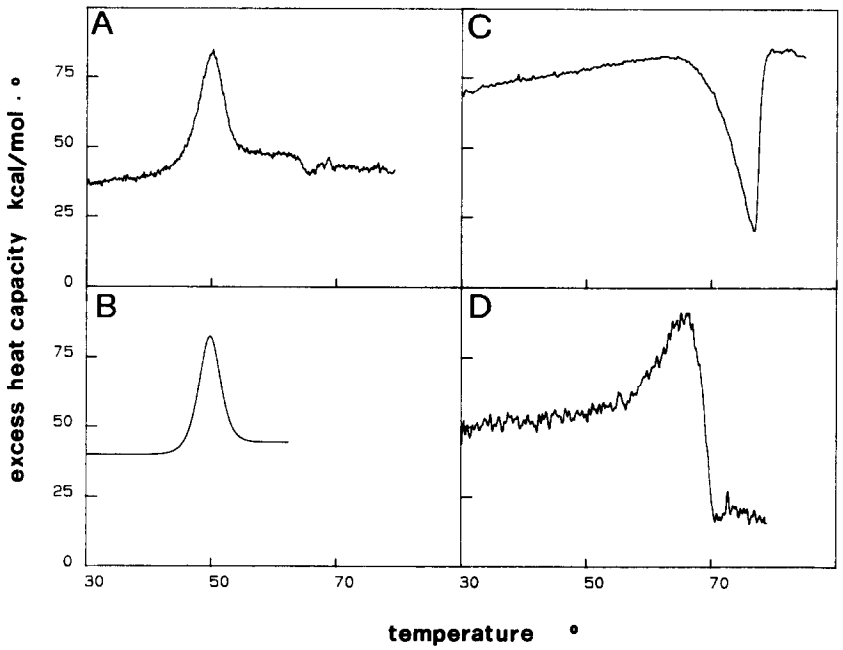

Fig. 2. Temperature dependence of molar partial heat capacity of PAO. Experimental conditions: (A) 0.5 M K-Pi buffer, pH 6, scanning rate 30° hr^{-1}; (B) calculated curve obtained by deconvolution of (A); (C) 0.5 M K-Pi, 5 mM spermidine, pH 6, scanning rate 60° hr^{-1}; (D) 0.2 M K-Pi. pH 5, scanning rate 30° hr^{-1}.

5°. A loss of catalytic activity of *ca* 5% after each preparative cycle was observed.

Δ^1-Pyrroline was also prepared by acid hydrolysis of 4-aminobutyraldehyde diethylacetal, according to ref. [27]. To a portion of 0.003 mol of the 4-aminobutyraldehyde diethylacetal, 5 ml H_2O and 1.6 ml 2 M HCl were added, and the soln incubated for 20 min at room temp. The 4-aminobutyraldehyde produced (yield 100%) was determined according to the method of ref. [28] by using 2-aminobenzaldehyde.

pH of optimum stability and calorimetric analysis. The enzyme was incubated for 1 hr at 55 and 64° with 2 ml 0.1 M K-Pi buffer in the pH range 3.5–7.0. Enzyme activity was determined every 5 min using 0.01 ml of enzyme in 2 ml of 0.2 M K-Pi, pH 6.5. Calorimetric scans were performed with a Microcal MC-2D high resolution differential scanning calorimeter equipped with the DA-2 digital data acquisition system. Thermograms were corrected by subtracting the instrumental base line obtained with both cells filled with the buffer used. Integration and deconvolution procedures were carried out using software provided by Microcal.

Sequence determination. N-Terminal sequence was determined with an Applied Biosystems 470 A gas-phase protein sequencer equipped with an Applied Biosystems 120 A PTH analyser for the on-line detection of phenylthiohydantoin (PTH) amino acids. The native protein (50 μg) was dissolved in 30 μl of H_2O and dried onto a TFA-treated glass fiber filter coated with polybrene. Prior to sample application, the filter was subjected to three cycles of Edman degradation, using the Applied Biosystems 03RPRE program. Anilino thioazolinone derivatives of amino acids were automatically converted to PTH amino acids and injected into the on-line analyser for identification.

Acknowledgements—This research was supported by funds for scientific research from Ministero della Pubblica Istruzione, Italy.

REFERENCES

1. Smith, T. A. and Barker, J. H. A. (1988) in *Progress in Polyamine Research* (Zappia, V. and Pegg, A. E., eds), p. 573. Plenum Press, New York.
2. Smith, T. A. and Bickley, D. A. (1974) *Phytochemistry* **13**, 2437.
3. Suzuki, Y. and Yanagisawa, H. (1980) *Plant Cell Physiol.* **21**, 1085.
4. Federico, R., Alisi, C. and Forlani, F. (1989) *Phytochemistry* **28**, 45.
5. Hirasawa, E., Watanaba, H. and Suzuki, Y. (1986) *Phytochemistry* **25**, 1739.
6. Yanagisawa, H., Kato, A., Hoshiai, S., Kamiya, A. and Torii, N. (1987) *Plant Physiol.* **85**, 906.
7. Smith, T. A. (1983) in *Methods in Enzymology* Vol. 94 (Tabor, H. and Tabor, C. W., eds), p. 311. Academic Press, New York.
8. Federico, R., Alisi, C., Forlani, F. and Angelini, R. (1989) *Phytochemistry* **28**, 2045.
9. Tabor, C. W. and Tabor, H. (1984) *Ann. Rev. Biochem.* **53**, 749.
10. Smith, T. A. (1976) *Phytochemistry* **13**, 633.
11. Smith, T. A. (1977) *Phytochemistry* **16**, 1647.
12. Kaur-Sawhney, R., Flores, H. E. and Galston, A. W. (1981) *Plant Physiol.* **68**, 494.
13. Angelini, R. and Federico, R. (1989) *J. Plant Physiol.* **135**, 212.
14. Galston, A. W. and Kaur-Sawhney, R. (1982) in *Plant Growth Substances* (Wareing, P. F., ed.) p. 451. Academic Press, London.
15. Kolattukudy, P. E. (1981) *Ann. Rev. Plant Physiol.* **32**, 539.
16. Lamport, D. T. A. (1986) in *Molecular and Physiological Aspects of Plant Peroxidases.* (Greppin, H., Penel, C. and Gaspar, Th., eds), p. 199. University of Geneva, Switzerland.

17. Biggs, K. G. and Fry, S. C. (1987) in *Physiology of Cell Expansion During Plant Growth.* (Cosgrove, D. J. and Knievel, D. P., eds), p. 46. American Society of Plant Physiologists, Rockville, U.S.A.
18. Bachrach, U., Rabina, S., Loebenstein, G. and Eilon, G. (1965) *Nature* **208**, 1095.
19. Yamada, T., Palm, C. J., Brooks, B. and Kosuge, T. (1985) *Proc. Nat. Acad. Sci. U.S.A.* **82**, 6522.
20. Smith, T. A. (1972) *Phytochemistry* **11**, 899.
21. Fromm, H. J., (1975) *Initial Rate Enzyme Kinetics.* Springer, Berlin.
22. Privalov, P. L. (1979) *Adv. Protein Chem.* **33**, 167.
23. Privalov, P. L., Tiktopulo, E. I., Venyaminov, S. Yu., Griko, Yu., Makhatadze, G. I. and Khechinashvili, N. N. (1989) *J. Mol. Biol.* **205**, 737.
24. Rinaldi, A., Floris, G. and Finazzi-Agrò, A. (1982) *Eur. J. Biochem.* **127**, 417.
25. Andrews, P. (1970) *Meth. Biochem. Anal.* **18**, 1.
26. Sawicki, E., Hauser, T. R., Stanley, T. W. and Elbert, W. (1961) *Anal. Chem.* **33**, 93.
27. Asakura, T., Takada, K., Ikeda, Y. and Matsuda, M. (1988) *Jikeikai Med. J.* **35**, 23.
28. Yamada, H., Kumagai, H., Kawasaki, H. and Ogata, K. (1967) *Biochem. Biophys. Res. Commun.* **29**, 723.