Pergamon

0031-9422(95)00980-9

Phytochemistry, Vol. 42, No. 2, pp. 291–293, 1996

0031-9422/96 $15.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF DIAMINE OXIDASE FROM *TRITICUM AESTIVUM* SHOOTS

YONEZO SUZUKI*

Laboratory of Biological Science, Kyoritsu Women's College, 2-2-1, Hitotsubashi, Chiyoda-ku, Tokyo 101, Japan

(*Received in revised form* 6 *November* 1995)

Key Word Index—*Triticum aestivum*; Gramineae; wheat; wheat seedlings; diamine oxidase; enzyme purification.

Abstract—Diamine oxidase (DAO; EC 1.4.3.6) from the shoots of wheat seedlings was separated into two fractions; A (M_r 55×10^4) and B (M_r 11×10^4) by chromatography on DEAE-Sephadex A 25. However, fraction A is probably related to fraction B. The enzyme was purified to apparent homogeneity by chromatography on DEAE-cellulose, DEAE-Sephadex A 25, hydroxylapatite and Sephacryl s-300. The SDS gel electrophoresis yielded a single band at M_r 5.8×10^4. 1,3-DAP (diaminopropane) was the best substrate of the enzyme. The K_m values for 1,3-DAP and for putrescine were 250 μM and 435 μM, respectively. Enzyme activity was strongly inhibited by the reagents for sulfydryl, copper-binding and carbonyl radical.

INTRODUCTION

Copper-containing plant DAOs are widespread in dicots [1, 2] and are particularly active in legumes [1–5]. Although DAOs have been detected in several species of the Gramineae [6–8], the enzyme has been purified and characterized only in rice [6], barley [7] and maize [8]. However some properties of maize DAO differ from those of the enzymes from rice and barley. This paper describes the purification of DAO from wheat shoots and some of its properties.

RESULTS AND DISCUSSION

The purification of wheat DAO is summarized in Table 1. Almost all the enzyme was adsorbed on the DEAE cellulose column and was eluted 0.2 M K-Pi buffer (pH 7) as in the case of maize DAO [8] (step 3). Although the enzyme was separated into fraction A (55×10^4 of M_r) and B (11×10^4 of M_r) by chromatography on DEAE-Sephadex A 25 (step 4), fraction A in 0.5 M KCl (kept overnight at 4°) has M_r of 11×10^4, suggesting that fraction A associates at relatively low ionic strength. Using a SDS gel electrophoresis system, a single band of M_r 5.8×10^4 was detected, suggesting that the wheat DAO consisted of two identical subunits. Among the tested amines (all at 17 mM), putrescine was most readily oxidized by wheat DAO. However at a lower concentration (6.5 mM) 1,3-DAP was more readily oxidized than other amines such as putrescine, agmatine and cadaverine in decreasing order (85%, 41% and 26%, respectively). Similar results were obtained by DAOs from the shoots of maize, rice and oat (unpublished data). The K_m values of wheat DAO as determined using the Lineweaver-Burk plot were 250 μM for 1,3-DAP and 435 μM for putrescine, indicating that 1,3-DAP has a much greater affinity than putrescine for the wheat DAO. The enzyme activity (under pH 7.0) for putrescine was inhibited 100% and 90% by *p*-CMPSA and *N*-EM (each 1 mM), respectively, as found in maize DAO [8]. The inhibition of wheat DAO by these compounds was not dependent on the incubation time and was uncompetitive. Meanwhile 10 mM Hg^{2+} inhibited rice DAO by 80% [6]. On the other hand, wheat DAO was inhibited by *o*-phenanthroline and 2,2′-dipyridyl (each 71% and 74% at 1 mM, respectively). Using the oxygen electrode, phenylhydrazine and diethyldithiocarbamate were also found to be strong inhibitors (each 88% and 90% at 0.1 mM, respectively) of wheat DAO. Cyclohexylamine (a strong inhibitor of soybean DAO [4]) inhibited wheat DAO to a lesser extent (5% at 1 mM).

EXPERIMENTAL

Plant. Wheat (*Triticum aestivum* L., cv norin No. 61) was grown for 6 days in moist vermiculite in plastic trays at 25° in total darkness.

Chemicals. The following were used: DEAE cellulose (Wako); DEAE-Sephadex A 25, Sephacryl s-300, Superose 12 HR 10/30 column and PD-10 column (Pharmacia). *p*-Chloromercuriphenylsulphonic acid (Na) (*p*-CMPSA), *N*-ethylmaleimide (*N*-EM), 1,3-dia-

*Present address: Katabami Botanical Laboratory, 759, Koyasu, Isehara-shi, Kanagawa-ken, 259-11 Japan.

Table 1. Purification of diamine oxidase from wheat shoots

Step	Total protein (mg)	Total activity (nkat)	Specific activity (pkat mg^{-1} protein)	Recovery (%)
1. Crude extract	3360	38.3	11.4	100
2. 70% $(NH_4)_2SO_4$ precipitate	2130	25.7	12.1	67
3. DEAE cellulose chromatography	420	11.6	27.7	30
4. DEAE-Sephadex A 25 chromatography	A: 13 B: 260	0.036 7.4	2.5 28.6	 19
5. Hydroxylapatite chromatography	A: 0.35 B: 4.25	0.063 1.6	181 319	 4
6. Sephacryl s-300 chromatography	B: 0.24	0.091	380	0.2

Enzyme activity was determined by method 1.

minopropane·2HCl (1,3-DAP) and horseradish peroxidase (Type II) (Sigma); agmatine sulphate (Aldrich); hydroxylapatite (Bio Gel HTP) (Bio-Rad). Other chemicals were obtained as pure commercial products.

Enzyme assays. DAO activity was determined by the following two methods depending on the object of the experiment; method 1 was based on the quinoneimine dye formation [8, 9], while method 2 was based on O_2 consumption [10]. These assay conditions were similar to those described in ref. [8].

Purification. All the operations were carried out 4–5°. *Step 1.* Six-day-old shoots of wheat seedlings (*ca* 500 g) were homogenized in a Waring blendor with 3 vol. (w/v) of chilled 0.1 M K-Pi buffer (pH 7) containing 10 mM $Na_2S_2O_5$. The homogenate was filtered and centrifuged at 20 000 *g* for 15 min. The supernatant was designated as crude extract. *Step 2.* The crude extract was made 0.7 satd with solid $(NH_4)_2SO_4$ with stirring overnight. The ppt. was dissolved in 200 ml of 20 mM K-Pi buffer (pH 7) and dialysed against 5 l of the same buffer overnight. The insoluble material was removed by centrifugation. *Step 3.* The supernatant of step 2 was applied to a DEAE cellulose column (4 × 15 cm) equilibrated and washed with 20 mM K-Pi buffer (pH 7) until the eluate showed no further *A* at 280 nm. The enzyme was then eluted with 0.2 M K-Pi buffer (pH 7). The active frs were collected and diluted with an equal vol. of H_2O. *Step 4.* The diluted enzyme soln was applied on the DEAE-Sephadex A 25 column (2 × 38 cm) equilibrated with 0.1 M K-Pi buffer (pH 7) and the effluent was collected (Fr. B). *Step 5.* The enzyme (fr. A) on the DEAE-Sephadex A 25 column was eluted with 0.2 M K-Pi buffer (pH 7). The active frs were collected and diluted with an equal vol. of H_2O as before. It was then applied to a hydroxylapatite column (2.5 × 4 cm) equilibrated with 0.1 M K-Pi buffer (pH 7). After washing with the same buffer until the eluate showed no further *A* at 280 nm, the enzyme was eluted from the column using 0.2, 0.3 and 0.4 M K-Pi buffer (pH 7). The most active frs (usually at 0.2 M) were collected. Meanwhile the effluent of step 4 was applied to another hydroxylapatite column (2.5 × 4 cm). After washing the column with the same buffer as before, the enzyme (fr. B) was eluted with 0.2 M K-Pi buffer (pH 7). *Step 6.* The active frs of step 5 of fr. B were collected and concd (using a collodion bag) to *ca* 1 ml and applied to a Sephacryl s-300 column (1.6 × 90 cm) equilibrated with 0.1 M K-Pi buffer (pH 7) containing 0.5 M KCl. The enzyme was fractionated with the same buffer. The active frs were combined and used as the purified enzyme.

M_r *of the enzyme.* To determine the M_r of wheat DAO, fr. A from step 5 and step 6 (fr. B) was applied to the Sephacryl s-300 column (1.6 × 90 cm) equilibrated with 0.1 M K-Pi buffer (pH 7) containing 0.5 M KCl, separately. Superose 12 HR 10/30 column was also used for the estimation of M_r using a Shimadzu GE-LC system G1 [8]. Thyroglobulin (M_r 669 k), aldolase (M_r 158 k), ovalbumin (M_r 43 k) and cytochrome c (M_r 12.4 k) were used to calibrate the column. SDS-PAGE was carried out according to ref. [11]. The purified enzyme (*ca* 30 μg) was previously treated with 1% SDS and 2% 2-mercaptoethanol at 100° for 5 min. Phosphorylase b (M_r 94 k), BSA (M_r 67 k), ovalbumin (M_r 43 k), carbonic anhydrase (M_r 30 k), trypsin inhibitor (M_r 20.1 k) and α-lactalbumin (M_r 14.4 k) (Pharmacia LMW calibration kit) were used as the marker proteins. *Protein* content was measured by the method of Ref. [12].

Acknowledgements—The author is grateful to Dr E. Hirasawa of the Department of Biology, Osaka City University for useful discussion and Mrs M. Hagiwara for some technical assistance.

REFERENCES

1. Suzuki, Y., Hirasawa, E., Yanagisawa, H. and Matsuda, H. (1990) in *Polyamines and Ethylene: Biochemistry, Physiology and Interactions* (Flores, H. E., Arteca, R. N. and Shannon, J. C., eds), p. 73. American Society of Plant Physiologists, Rockville, MD.
2. Medda, R., Padiglia, A. and Floris, G. (1995) *Phytochemistry* **39**, 1.

3. Padiglia, A., Cogoni, A. and Floris, G. (1991) *Phytochemistry* **30**, 3895.
4. Vianello, F., Di Paolo, M. L., Stevanato, R., Gasparini, R. and Rigo, A. (1993) *Arch. Biochem. Biophys.* **307**, 35.
5. Hagiwara, M., Soeda, M. and Suzuki, Y. (1994) *J. Plant Physiol.* **144**, 12.
6. Chaudhuri, M. M. and Ghosh, B. (1984) *Phytochemistry* **23**, 241.
7. Cogoni, A., Piras, C., Farci, R., Melis, A. and Floris, G. (1990) *Plant Physiol.* **93**, 818.
8. Suzuki, Y. and Hagiwara, M. (1993) *Phytochemistry* **33**, 995.
9. Smith, T. A. (1993) in *Methods in Plant Biochemistry* 8, p. 17.
10. Estabrook, R. W. (1967) in *Methods in Enzymology* (Estabrook, R. W. and Pullman, M. E., eds) Vol. 10, p. 41. Academic Press, London.
11. Laemmli, U. K. (1970) *Nature* **227**, 680.
12. Kalcker, H. M. (1947) *J. Biol. Chem.* **167**, 461.