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CARBONIC ANHYDRASES FROM LEAVES AND ROOTS OF *DAUCUS CAROTA*

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Key Word Index—*Daucus carota*; Apraieae; wild carrot; carbonic anhydrase; caffeine; nicotine.

Abstract—Carbonic anhydrases (CAs) (carbonate hydrolase; EC 4.2.1.1) from leaves and roots of mature *Daucus carota* cv. *nantes* were purified and characterized. The purification levels were 75- and 73-fold in leaves and roots, respectively. The optimum temperature for the enzymes from both sources was 75°. pH optima were 8.5 and 9.0 in leaves and in roots, respectively. Each enzyme molecule was a hexamer having M_r 137 800 and the subunit M_r was 22 800. A dimer CA which has a M_r of 45 700 was also detected. The amount of this dimer was higher in leaves than in roots. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

It is known that carbonic anhydrases (CAs) are products of a gene family that encodes seven isoenzymes [1]. However, most interest has been focused on human CA I and CA II (HCA I and HCA II) and bovine CA III which are monomers with M_r values of *ca* 29 000 [2–5]. The structure, enzymic function and physiological role of mammalian CAs from various sources, such as bovine erythrocytes [6], man [7, 8], rhesus monkey [9] and horse [10], have been studied extensively. The M_r values of CAs of vertebrates are 29 000–30 000, and each enzyme molecule contains one atom of zinc which is necessary for activity [11]. The CA in plants, first detected in 1939 [12], has been partially purified and characterized from various sources [13–15].

The CAs from higher plants exist in at least two electrophoretically separable types. One type, found predominantly in monocots, has a M_r of 40 000 [16]. The other, isolated from dicots, is a hexameric enzyme with a M_r of 180 000 and contains six tightly bound zinc ions [15–18]. This value is different from those in human kidney and rabbit erythrocytes (M_r 66 000 and 54 000, respectively) [1, 19]. However, there has so far been no report on the CAs in *Daucus carota*. The purpose of the present study was to purify the CAs from the leaves and roots of *D. carota* cv. *nantes*, to characterize them and to compare their properties with those of mammalian and other plants CAs.

RESULTS AND DISCUSSION

The CAs of leaves and roots of *D. carota* were purified by DEAE–cellulose ion exchange chromatography. The amount of purified CA was higher in leaves than in roots while CA activity was higher in roots than leaves. However, when the amounts and activities of the two enzymes were compared, there was no marked difference between them. Esterase activity of CA was detected with *p*-nitrophenyl acetate as substrate. In an earlier report, it was noted that CA from parsley and spinach showed no esterase activity [15]: this could be due to the fact that the purification levels of the CAs in leaves and roots of *D. carota* are higher than those for spinach or parsley. In the present study, CA of leaves was purified 75- and 73-fold in roots (Table 1). However, the CA of parsley has previously been purified 12-fold [15]. The other factor might be that the buffer solution used in the purification of CA from parsley was performed in 0.1 M *N*-ethylnaphthalene acetate/0.1 M 2-mercaptoethanol, pH 7, in which we could not detect esterase activity in CA of leaves or roots of *D. carota*. *N*-Ethylnaphthalene acetate may partially inhibit the hydrolysis of *N*-ethylnaphthalene acetate by CA. However, in the present study, when this buffer was replaced with 0.1 M Tris–acetate/0.01 M 2-mercaptoethanol, pH 7, esterase activity was detected. Therefore, this buffer solution may be the most appropriate one for enzyme elution and for storage of enzyme. The third possibility is that the amino acid sequence of CA isolated from *D. carota* leaves and roots differs from that of the CA in spinach or parsley leaves and roots. For each CA of *D. carota* (leaves and roots),

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Table 1. Carbonic anhydrase from *Daucus carota* cv. *nantes* leaves and roots

Enzyme fraction	Volume (ml)	Activity (EU ml^{-1})	Total activity		Protein (mg ml^{-1})	Specific activity (EU mg^{-1})	Purification (fold)
			EU	%			
Leaves							
Crude extract	1000	1.21	1.21	100	32.3	3.7×10^{-2}	–
(NH)$_2$SO$_4$ 120 g	950	0.99	936	78	27.7	3.5×10^{-2}	0.95
(NH)$_2$SO$_4$ 180 g	870	0.87	759	81	18.3	4.7×10^{-2}	1.34
After DEAE–cellulose column	200	0.78	157	21	0.22	3.56	74.76
Roots							
Crude extract	1000	1.0	995	100	37.2	2.6×10^{-2}	–
(NH)$_2$SO$_4$ 120 g	920	0.81	741	74	30.7	2.6×10^{-2}	0.98
(NH)$_2$SO$_4$ 180 g	800	0.72	572	77	22.5	3.2×10^{-2}	1.209
After DEAE–cellulose column	175	0.63	109	19	0.27	2.32	73.02

V_{max} and K_{m} values were 0.65 mM and 1.57 mM for esterase activities, respectively. These values are compatible with each other. The optimum pH values of the CAs from *D. carota* were 9.0 and 8.5 in leaves and in roots, respectively. CA has activity in the pH range 8–10, which is higher than that for the CA of parsley, which has an optimum pH of 6.8 [15].

The optimum temperature for both leaf and root CA was 75°. The temperature range at which enzymes have activity was 40–80°. This range is higher than the CA of parsley, which has previously been reported to have activity between 40° and 60° [15]. This could suggest that high temperature has a positive effect on the CA of leaves and roots of *D. carota*.

The M_r of the CAs from both sources determined by gel filtration chromatography was 137 800. This is close to the M_r of the CA of spinach, (140 000) [14], but lower than that of parsley (180 000) [15]. On 3–10% SDS electrophoresis in which bovine CA was used as standard, the M_r of each subunit was *ca.* 22 800. However, the M_r of a CA dimer was 45 700 (Fig. 1). The ratio of the M_r of the enzyme to M_r of the subunit indicates that the CA was a hexamer. It has been reported previously that the CA of spinach with M_r 140 000 is a hexameric enzyme which has no Zn^{2+} ion [14]. Thus, we suggest that the CAs obtained from leaves and roots of *D. carota*, which have M_r values near to that of spinach CA, possibly do not contain Zn^{2+}.

The results of the inhibition studies were surprising; chemical reagents such as caffeine, nicotine, nicotine amide, SCN^- sulphanilamide and acetazolamide [25], which are known as *inhibitors* of mammalian CA, were in fact *activators* of the CA enzymes from leaves and roots of *D. carota* (Figs 2 and 3).

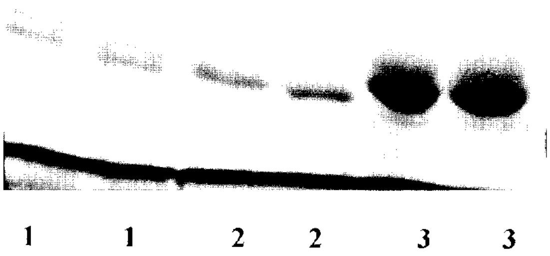

Fig. 1. Electrophoresis pattern of *Daucus carota* cv. *nantes* carbonic anhydrases: (1) leaves; (2) roots; (3) borine carbonic anhydrase.

EXPERIMENTAL

Extract preparation. *Daucus carota* cv. *nantes* was collected from East Anatolia, Turkey, and the leaves and roots of the plant were separated mechanically. They were stored at −31°. Separated leaves and roots were chopped with a blender in the presence of 2 l of buffer (0.05 M NaPi, 0.01 M 2-mercaptoethanol, pH 7) for each kg of sample. The suspensions were filtered twice through a fibreglass window screen. The filtrates were centrifuged in a continuous flow and the ppt was discarded. The pulps were squeeze dried and discarded. Afterwards, 180 g solid $(NH_4)_2SO_4$ was added per 1 g of supernatant. The mixtures were stirred for 1 hr at 4°, and then centrifuged for 5 min at 10 000 rpm. Suspensions were filtered through a filter pad and were first dialysed against 5 changes of H_2O for 5 hr and subsequently against 2 l of the buffer (0.1 M Tris–acetate, 0.01 M 2-mercaptoethanol, pH 7) for 3 hr. The resulting soln was then cleared of insoluble materials by centrifugation for 20 min at 8000 rpm at 0° [15].

Enzyme purification. Enzymes present in extracts were purified by ion exchange chromatography on a 3 × 50 cm column that contained DEAE–cellulose.

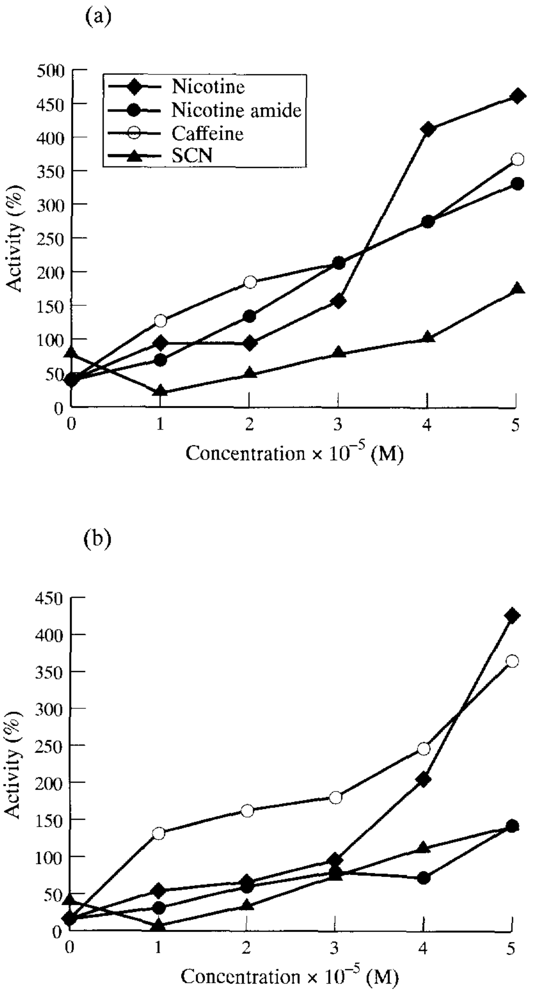

Fig. 2. Effect of nicotine, nicotine amide, caffeine and KSCN on the activity of purified carbonic anhydrase enzyme from *Daucus carota* cv. *nantes*: (a) leaves; (b) roots.

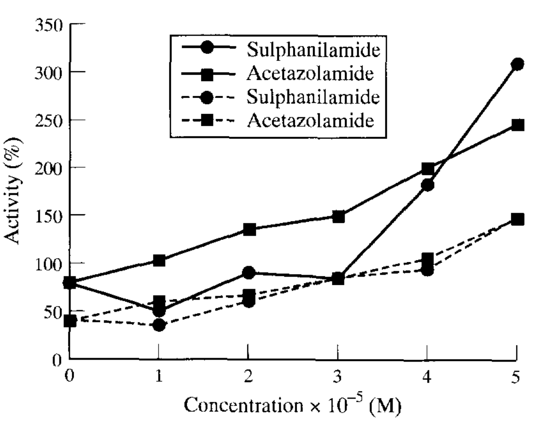

Fig. 3. The effect of sulphanilamide and acetazolamide on carbonic anhydrase enzymes activity from *Daucus carota* cv. *nantes*: (——) leaves; (- - - -) roots.

Elution was carried out with 0.2 M Tris–acetate, 0.01 M 2-mercaptoethanol, pH 7.

Protein determination. Elution fractions having *A* at 280 nm were pooled and the amount of protein was determined as described in ref. [20].

Enzyme activity determination. Esterase activity was determined as described in ref. [21]. Briefly, 1.5 ml of buffered enzyme (1 ml 0.1 M Tris–acetate, 0.01 M 2-mercaptoethanol, pH 7, and 0.5 ml purified enzyme of concn 10 mg ml^{-1}), and 1.5 ml of *p*-nitrophenyl acetate (3 mM) were mixed, and after 3 min *A* was read at 348 nm. V_{max}, K_m and optimum pH were determined. While substrate vol. was increased (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 ml) the vol. of enzyme was fixed 1 ml and buffer was added to make up a total vol. of 3 ml in determining V_{max} and K_m values. V_{max} and K_m values were determined from a Lineweaver–Burk graph. Hydrolysis activity of purified enzymes was measured by determining the time necessary to change the pH from 8.2 to 6.3 [22]. Enzyme units were calculated according to the formula: 1 U = $(t_o - t_c)/t_c$, where t_0 and t_c are the time (sec) needed for the pH change without and with enzyme reactions, respectively.

The effect of various inhibitors on enzyme activity. Hydrolysis activities of purified enzymes in the presence of caffeine, nicotine, nicotine amide, KSCN, sulphanilamide and acetazolamide which are known as inhibitors were measured [22].

SDS–PAGE electrophoresis. Electrophoresis was carried out on 3–10% SDS–PAGE gel as described in ref. [23]. Human CA I and bovine carbonic anhydrase were purified by affinity chromatography and used as standards [24].

Determination of M_r. M_r of purified carbonic anhydrase enzymes of leaves and roots of *D. carota* were determined by using Sephadex-G 150. A mixture of standard proteins each of which had a concn. of 0.2 mg ml^{-1} was applied on the column. Purified CA was added to the equilibrated column and eluted with 0.05 M NaP$_i$/1 mM ditihioerythritol, buffer, pH 7.

REFERENCES

1. Sly, W. W. and Hu, P. Y., *Annual Reviews in Biochemistry*, 1995, **64**, 375.
2. Kannan, K. K., Ramanadham, M. and Jones, T. A., *Annals of the New York Academy of Science*, 1984, **429**, 49.
3. Eriksson, A. E., Jones, T. A. and Liljas, A., *Proteins*, 1988, **4**, 274.
4. Hakansson, K., Carlsson, M., Svensson, L. A. and Liljas, A. J., *Molecular Biology*, 1992, **227**, 1192.
5. Eriksson, A. E. and Liljas, A., *Proteins*, 1993, **16**, 29.
6. Lindskog, S., *Biochimica et Biophysica Acta*, 1960, **39**, 218.
7. Nyman, P. O., *Biochimica et Biophysica Acta*, 1961, **52**, 1.
8. Laurent, G., Castay, M., Marriq, C., Garcon, D., Charrel, M. and Derrien, Y., *Bulletin de la Societe Chimique Biologique*, 1965, **47**, 1101.

9. Duff, T. A. and Coleman, J. E., *Biochemistry*, 1966, **5**, 2009.
10. Furth, A. J., *Journal of Biological Chemistry*, 1968, **243**, 4832.
11. Lindskog, S. and Malmstrom, B. G., *Journal of Biological Chemistry*, 1962, **237**, 1129.
12. Neish, A. C., *Biochemical Journal*, 1939, **33**, 300.
13. Waygood, E. R. and Clendenning, K. A., *Science*, 1951, **113**, 177.
14. Kondo, K., Yonezawa, T. and Chiba, H., *Bulletin of the Research Institute of Food Science*, 1952, **8**, 1, 17, 28.
15. Tobin, A. L., *Journal of Biological Chemistry*, 1970, **245**, 2656.
16. Tobin, A. L., *NASA SP*-188. NASA, Washington, D.C., 1968, p. 139.
17. Kisiel, W. and Graf, G., *Phytochemistry*, 1966, **5**, 2641.
18. Pocker, Y. and Ng, J. S. Y., *Proceedings of the International Congress on Biochemistry*, 1973, **9**, 64.
19. Pocker, Y. and Ng, J. S. Y., *Biochemistry*, 1973, **12**, 5127.
20. Bradford, M. M., *Analytical Biological Chemistry*, **72**, 248.
21. Verpoorte, J. A., Mehta, S. and Edsall, J. T., *Journal of Biological Chemistry*, 1967, **242**, 4221.
22. Rickli, E. E., Ghazanfar, S. A. S., Gibbons, B. H. and Edsall, J. T., *Journal of Biological Chemistry*, 1964, **239**, 1065.
23. Laemmli, U. K., *Nature*, 1970, **227**, 680.
24. Demir, N., Küfrevioğlu, Ö. İ., Keha, E. E. and Bakan, E., *Biofactors*, 1993, **4**, 129.
25. Maren, T. H. and Couto, E. D., *Archives of Biochemistry and Biophysics*, 1979, **196**, 501.