Phytochemistry, 1975, Vol. 14, pp. 383–386. Pergamon Press. Printed in England.

HYDROLYSIS OF 3′,4′-DICHLOROPROPIONANILIDE BY AN ARYL ACYLAMIDASE FROM *TARAXACUM OFFICINALE*

ROBERT E. HOAGLAND*

Department of Biochemistry, North Dakota State University, Fargo, N.D. 58102, U.S.A.†

(*Received* 29 *May* 1974)

Key Word Index—*Taraxacum officinale*; Compositae; dandelion; herbicide metabolism; aryl acylamidase; propanil; 3′,4′-dichloropropionanilide hydrolysis.

Abstract—An aryl acylamidase (aryl-acylamine amidohydrolase, E.C. 3.5.1.a) which hydrolyzes the herbicide propanil (3′,4′-dichloropropionanilide), was isolated from dandelion roots and partially purified and characterized. Specificity tests on the enzyme revealed that it could hydrolyze various chlorine ring-substituted propionanilides and 3,4-dichloroanilide alkyl compounds. The partially purified enzyme was inhibited by several sulfhydryl reagents and metal ions. The pH optimum was broad, between 7·4 and 7·8. The apparent activation energy, determined from an Arrhenius plot, was 9·0 kcal/mol (37700 J/mol) for the hydrolysis of 3′,4′-dichloropropionanilide. The apparent K_m was $1{\cdot}7 \times 10^{-4}$ M with propanil as substrate.

INTRODUCTION

Propanil (3′,4′-dichloropropionanilide) is used as a post-emergence herbicide to control barnyardgrass and several broadleaf weeds in rice fields [1–3]. This compound has also been used post-emergence on tomatoes [4]. Microorganisms [5–7] and mammalian liver tissue [8] have enzymes which can degrade propanil by hydrolysis of the amide bond in the molecule. Early investigations of the metabolism of this herbicide in plants showed that rice seedlings could rapidly transform propanil to 3,4-dichloroaniline and propionic acid [9, 10]. An aryl acylamidase that could metabolize propanil in this manner was isolated from rice seedlings and partially purified and characterized [11]. Other aryl acylamidases which hydrolyze propanil have been reported to occur in various weeds [12, 13] and in several agronomic and horticultural plants [14]. A tulip aryl acylamidase that can hydrolyze propanil has also been partially purified and characterized [15]. In this paper the partial purification and properties of an aryl acylamidase from dandelion roots are reported.

* Present address: U.S. Department of Agriculture, Agricultural Research Service, Southern Weed Science Laboratory, Stoneville, MS 38776, U.S.A.

† Published with the approval of the Director, North Dakota Agricultural Experiment Station, Journal Article No. 501.

RESULTS AND DISCUSSION

Effect of gravitational fields

Crude root homogenates were centrifuged for 2 hr at 30000, 70000 and 150000 *g* and after centrifugation the supernatant lost 0, 3, and 22% of the total activity respectively. It therefore appears that the dandelion root enzyme may be associated at least in part with cell membrane fragments. The aryl acylamidase preparation from tulip which hydrolyzes propanil showed no activity loss from the supernatant when centrifuged at 150000 *g* for 90 min. In contrast, the rice aryl acylamidase [11] lost 80% of the activity from the supernatant when centrifuged at 150000 *g* for 2 hr. Several plants have been shown to contain both soluble and particulate aryl acylamidases [12]. An aryl acylamidase from rat liver that hydrolyzed propanil is a particulate enzyme [8]. A tulip enzyme that hydrolyzes propanil does not appear to be associated with particulate material [15].

Isolation and partial purification of enzyme

The isolation and partial purification of the dandelion root aryl acylamidase was achieved by extraction of root tissue with buffer, ammonium sulfate precipitation, and Sephadex G100 gel filtration (see Experimental). The crude enzyme preparation had *ca* 360 enzyme units for 10 g fr wt.

This is about 10% of the total amount of enzyme units present in tulip bulbs [15]. The purification steps yielded an enzyme purification of about 8-fold with 89% recovery. This partially purified enzyme was used in all of the characterization studies reported here. Aryl acylamidase activity with propanil as substrate is also present in dandelion leaves [13].

Effect of pH on the enzyme activity

The pH optimum of the partially purified dandelion aryl acylamidase was very broad, between 7·4 and 7·8 with 70 and 84% of maximum activity (pH 7·5) at pH 6·2 and 8·8 respectively. High enzyme activity was also observed over a wide range of pH values above the pH optimum. The pH optima were very similar for the rice [11] and tulip [15] aryl acylamidases. Both of these enzymes showed high activity over a broad range of pHs. Aryl acylamidases from chicken [16] and rat kidney [17] also possess similar pH optima.

Stability of dandelion enzyme at various temperatures

The partially purified enzyme was exposed to various temperatures for 5 min prior to assay at 35°. It was noted that the enzyme retained high activity only up to a 5 min exposure at about 45°. The enzyme was inactivated 50% on heating for 5 min at 46–48°. A 5 min exposure at 68° totally inactivated the enzyme. The tulip aryl acylamidase had a higher temperature stability [15].

Effect of assay temperature on aryl acylamidase activity

The optimum temperature for assay with 3',4'-dichloropropionanilide as substrate was 35°. There was essentially no hydrolysis of propanil at assay temp. above 50°. From an Arrhenius plot, an apparent activation energy of 9·0 kcal/mol (37 700 J/mol) was determined for the hydrolysis of propanil. The apparent activation energy for the tulip aryl acylamidase was 10·3 kcal/mol [15].

Inhibition studies on dandelion aryl acylamidase

The effects of several metal ions and sulfhydryl reagents on the partially purified dandelion enzyme are shown in Table 1. Propanil was used as substrate. Copper (II) and mercury (II) ions were highly inhibitory to the enzyme activity, while cobalt (II) gave only 15% inhibition. Catechol showed the greatest inhibition of the organic compounds tested; 73%. *p*-Chloromercuribenzoic acid and *p*-benzoquinone gave 61% and 50% inhibition, respectively. The sulfhydryl reagents, *o*-iodosobenzoic acid, *N*-ethylmaleimide and iodoacetic acid showed low inhibition. These latter three compounds showed significant inhibition of the

Table 1. Inhibition studies on *Taraxacum officinale* aryl acylamidase

Inhibitor	Concn (mM)	Inhibition (%)
$HgCl_2$	0·5	89
$CuCl_2$	0·5	86
$CoCl_2$	1·0	16
$FeCl_3$	1·0	6
Catechol	0·5	73
p-Chloromercuri benzoic acid	0·25	61
p-Benzoquinone	0·50	50
o-Iodosobenzoic acid	0·25	27
N-ethylmaleimide	1·0	23
Iodoacetic acid	1·0	2

The complete reaction mixture contained 150 μmol phosphate buffer, pH 7·5, 0·8 μmol substrate, 250 μg Sephadex G100 enzyme and de-ionized H_2O to a total vol of 3 ml. The inhibitors were incubated with the enzyme, buffer and water for 10 min prior to substrate addition. The reaction was incubated at 35°, 4 hr, and then assayed according to the standard procedure.

Table 2. Substrate specificity of *Taraxacum officinale* enzyme on various chlorine ring-substituted propionanilides

Substrate	Hydrolysis product (nmol)	Relative activity (%)
3′,4′-Dichloropropionanilide	19·2	100
4′-Chloropropionanilide	10·4	54
3′-Chloropropionanilide	9·8	51
2′,4′-Dichloropropionanilide	9·4	49
2′-Chloropropionanilide	7·1	37
2′,3′-Dichloropropionanilide	2·7	14
2′,6′-Dichloropropionanilide	0·0	0

The complete reaction mixture contained 150 μmol phosphate buffer, pH 7·5, 0·8 μmol substituted anilide, 250 μg Sephadex G100 enzyme and de-ionized H_2O to a total vol of 3 ml. The mixture was incubated at 35°, 4 hr, and then assayed to determine the extent of hydrolysis.

rice enzyme [11] but only low inhibition of the activity of the tulip aryl acylamidase [15]. Rat kidney, rat liver and chicken kidney aryl acylamidases have been shown to be strongly inhibited by several sulfhydryl reagents [15, 17].

Substrate specificity

Substrate specificity tests on the dandelion root aryl acylamidase were made with a variety of chlorine ring-substituted and 3,4-dichloroanilide alkyl analogs of propanil (Tables 2 and 3). The enzyme had a broad substrate specificity on mono- and di-chlorosubstituted propionanilides. The highest activity was exhibited with propanil as substrate (Table 2). The substrate specificity of the enzyme on several 3,4-dichloropropionanilides indicated a high substrate preference for propanil when compared to the other compounds tested (Table 3). The rice [11] and tulip [15] aryl acylamidases also showed a broad substrate specificity when tested with these compounds.

The dandelion enzyme did not hydrolyze amide bonds of fenuron, (3-phenyl-1,1-dimethylurea); monuron, (3-(4-chlorophenyl)-1,1-dimethylurea); CIPC, (isopropyl *N*-(3-chlorophenyl) carbamate) and IPC, (*N*-isopropyl phenylcarbamate). An enzyme from a soil microorganism was able to catalyze the hydrolysis of propanil and the two carbamates, IPC and CIPC [6]. The substrate specificity of the dandelion aryl acylamidase was somewhat different from that of the tulip enzyme [15] in that the former showed only minimal hydrolytic activity on 2′-nitroacetanilide [18].

Kinetic studies

Using the Fortran program of Cleland [19, 20] the apparent K_m was $1{\cdot}7 \times 10^{-4}$ M with propanil as substrate. The apparent K_m of the rice [11] and tulip [15] enzymes with propanil as substrate are $2{\cdot}93 \times 10^{-3}$ M and $2{\cdot}5 \times 10^{-3}$ M, respectively.

EXPERIMENTAL

Plant material. Common dandelion seeds (*Taraxacum officinale* Weber) were germinated in large pots containing a soil-vermiculite mixture and grown under greenhouse conditions for 8–10 months. Half-strength Hoagland's nutrient soln was added periodically. The roots were used as the source of enzyme.

Table 3. Substrate specificity of *Taraxacum officinale* enzyme on several 3,4-dichloroanilide alkyl analogs

Substrate	Hydrolysis product (nmol)	Relative activity (%)
3′,4′-Dichloropropionanilide	19·2	100
3,4-Dichloroacetanilide	9·4	49
3′,4′-Dichlorobutryanilide	7·1	37
3′,4′-Dichlorovaleranilide	2·3	12
3′,4′-Dichloro-2-methacrylanilide	1·5	8

The complete reaction mixture contained 150 μmol phosphate buffer, pH 7·5, 0·8 μmol substituted anilide, 250 μg Sephadex G100 enzyme and de-ionized H_2O to a total vol of 3 ml. The mixture was incubated at 35°, 4 hr, and then assayed to determine the extent of hydrolysis.

Extraction and purification of aryl acylamidase. Mature roots were washed with H_2O and blotted between filter papers. For each 10 g (fr. wt) 100 ml of 0·05 M phosphate buffer, pH 7·5 and 10 mM thioglycollate was added. The roots and buffer were homogenized in an electric blender for 2 min at high speed. This homogenate was filtered through 2 layers of cheesecloth and the filtrate was centrifuged at 30000 *g* for 20 min. The supernatant (crude homogenate) contained a total of 360 enzyme units with a sp. act. of 2·4. The protein fraction precipitating at 65% $[NH_4]_2SO_4$ saturation contained the bulk of the enzyme activity. The precipitated protein was collected by centrifugation at 10000 *g* for 10 min and the protein pellet was redissolved in phosphate buffer and placed on a Sephadex G100 gel filtration column for further purification. Eluate from the column was monitored at 280 nm and fractions were collected. The active aryl acylamidase fractions were pooled and used in the characterization tests. Purification, on the average, was about 8-fold over the crude homogenate (sp. act. 19·2). The yield was about 89%.

Aryl acylamidase activity. The standard enzyme reaction mixture contained 0·8 μmol substrate, 150 μmol K phosphate buffer, pH 7·5, enzyme, and deionized H_2O in a total vol. of 3 ml. The reaction was started by the addition of the enzyme. The reaction mixture was incubated at 35° for 4 hr unless otherwise stated. The reaction was terminated by the addition of 2 ml of an acid mixture consisting of 8 vol., M HCl and 1 vol. HOAc. Controls were prepared by adding the enzyme to the reaction mixture after 2 ml of the acid mixture had been added. The standard enzyme assays with propanil and the assays with propanil analogs were based on the formation of chloroaniline or aniline. The aniline produced from enzymatic hydrolysis was measured colorimetrically by a modified Bratton–Marshall reaction [15, 21]. The chloroanilines used as standard products were purified by recrystallization, sublimation or preparative TLC before use. The assay with 2′-nitroacetanilide as substrate is described elsewhere [18]. An enzyme unit is described here as the amount of enzyme required to hydrolyze 1 nmol of acyl anilide per hr under the assay conditions. Sp. act. is defined as the number of enzyme units per mg protein. Protein concn was determined by the method of Lowry *et al.* [22] with bovine serum albumin as the standard protein.

Reagents. Ring-substituted and alkyl-substituted anilides used as substrates were synthesized by the Huffman and Allen procedure [23]. High purity pesticides were obtained as follows: propanil from Rohm and Haas Company, Philadelphia, Pennsylvania; monuron and fenuron from E. I. duPont de Nemours, Wilmington, Delaware; CIPC and IPC from Pittsburgh Plate Glass Company, Pittsburgh, Pennsylvania.

Note. This work was presented in part at the 165th National Meeting of the American Chemical Society, Pesticide Chemistry Division, Dallas, Texas, April 1973.

Acknowledgements—The author wishes to thank George Graf for advice and suggestions and E. D. Handel for technical assistance during a portion of this work.

REFERENCES

1. Sarjas, P. De (1961) *Comptes Rendus des Journeés d'Etudes sur les Herbicides* p. 82, European Weed Research Council-Coluna, Paris.
2. Smith, R. J. (1961) *Weeds* **9,** 318.
3. Yamada, N. and Nakamura, H. (1963) *Proc. Crop Sci. Soc. Japan* **32,** 69.
4. *Herbicide Handbook of the Weed Society of America* (1970) p. 176, Humphrey Press, Geneva.
5. Bartha, R. and Pramer, D. (1967) *Science* **156,** 1617.
6. Kearney, P. C. (1965) *J. Agric. Food Chem.* **13,** 561.
7. Bartha, R. (1968) *J. Agric. Food Chem.* **16,** 602.
8. Williams, C. H. and Jacobson, K. H. (1966) *Toxicol. Appl. Pharmacol.* **9,** 495.
9. Unger, V. H., McRae, D. H. and Wilson, H. F. (1964) *Weed Soc. Abstr.* p. 86.
10. McRae, D. H., Yih, R. Y. and Wilson, H. F. (1964) *Weed Soc. Abstr.* p. 87.
11. Frear, D. S. and Still, G. G. (1968) *Phytochemistry* **7,** 913.
12. Ahatsuka, T. and Kasakura, N. (1972) *Ibarski Daigaku Nogakubu Gakujutsu Hokolu* **20,** 17.
13. Hoagland, R. E. and Graf, G. (1972) *Weed Sci.* **20,** 303.
14. Hoagland, R. E., Graf, G. and Handel, E. D. (1974) *Weed Res.* **14.**
15. Hoagland, R. E. and Graf, G. (1972) *Phytochemistry* **11,** 521.
16. Nimmo-Smith, R. H. (1960) *Biochem. J.* **75,** 284.
17. Mahadevan, S. and Tappel, A. L. (1967) *J. Biol. Chem.* **242,** 2369.
18. Hoagland, R. E. and Graf, G. (1971) *Enzymologia* **41,** 313.
19. Cleland, W. W. (1963) *Nature* **198,** 463.
20. Cleland, W. W. (1967) in *Advances in Enzymology* (Word, F. F., ed.) Vol. 29, p. 1, Interscience, New York.
21. Bratton, A. C. and Marshall, E. K. (1939) *J. Biol. Chem.* **128,** 537.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
23. Huffman, C. W. and Allen, S. E. (1960) *J. Agric. Food Chem.* **8,** 298.