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AN ARYL ACYLAMIDASE FROM TULIP WHICH HYDROLYZES 3',4'-DICHLOROPROPIONANILIDE

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Abstract—An aryl acylamidase which hydrolyzes the herbicide propanil (3',4'-dichloropropionanilide), has been isolated from tulip bulbs and partially purified and characterized. Several chlorinated ring-substituted propionanilide analogs as well as 3,4-dichloroacetanilide and 3',4'-dichloro-2-methacrylanilide (dicryl) were hydrolyzed by the enzyme. The partially purified enzyme lacked sensitive-SH groups and possessed a broad pH optimum between 6·8 and 7·8. The enzyme was relatively stable up to about 50°, but lost activity rapidly on exposure to temperatures above this. The optimum temperature of assay with 3',4'-dichloropropionanilide was 53°. The apparent activation energy of the enzyme was 10·3 kcal/mole and the apparent K_m was $2{\cdot}50 \times 10^{-3}$m, with 3', 4'-dichloropropionanilide as substrate. The properties of this acylamidase from tulip were compared with aryl acylamidases from rice and liver.

INTRODUCTION

PROPANIL (3',4'-dichloropropionanilide) is used as a herbicide to control weeds such as barnyard grass and various broadleaf weeds in cultivated rice fields. It also has application as a post-emergence herbicide in tomatoes.[1]

Metabolic studies of propanil carried out with bacterial, plant and mammalian systems, showed that 3',4'-dichloropropionanilide, is converted usually enzymically, to 3,4-dichloroaniline and other degradation products. Kearney[2] has purified and characterized an enzyme from *Pseudomonas striata* Chester which is able to hydrolyze propanil to 3,4-dichloroaniline and propionic acid. Bartha and Pramer[3] have also shown that propanil is transformed into 3,4-dichloroaniline by soil microorganisms. Williams and Jacobson[4] found enzymes in liver homogenates of rats, mice, rabbits and dogs which hydrolyzed propanil. Studies by Unger *et al.*,[5] McRae *et al.*,[6] and Still[7] showed that intact rice seedlings could rapidly metabolize 3',4'-dichloropropionanilide to 3,4-dichloroaniline and propionic acid. Recently, Frear and Still[8] have partially purified and characterized an aryl acylamidase from rice plants which is responsible for hydrolytic degradation of propanil.

In our studies on the metabolism of herbicides by higher plants, we have found an enzyme in tulip that also hydrolyzes propanil. In this paper the isolation, partial purification and some properties of this enzyme are reported.

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[1] *Herbicide Handbook of the Weed Society of America*, p. 117, Humphrey Press, Geneva, New York (1967).
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[4] C. H. WILLIAMS and K. H. JACOBSON, *Toxicol. Appl. Pharmacol.* **9**, 495 (1966).
[5] V. H. UNGER, D. H. MCRAE and H. F. WILSON, *Weed Soc. Abstr.* 86 (1964).
[6] D. H. MCRAE, R. Y. YIH and H. F. WILSON, *Weed Soc. Abstr.* 87 (1964).
[7] G. G. STILL, *Plant Physiol.* **43**, 543 (1968).
[8] D. S. FREAR and G. G. STILL, *Phytochem.* **7**, 913 (1968).

RESULTS AND DISCUSSION

Distribution of Tulip Aryl Acylamidase Which Hydrolyzes Propanil

A distribution study of the aryl acylamidase in the mature tulip plant showed (Table 1) that the bulbs possessed fifty times more enzyme units than either the stems or the leaves. The specific activity values indicate that a large portion of the bulb protein is active in propanil hydrolysis, whereas in the stems and leaves only a very small amount of the total protein functions as active aryl acylamidase. The number of units of the propanil-hydrolyzing enzyme in tulip bulbs is about the same as that in rice leaves, when compared on a fresh weight basis.[8] The specific activity of the tulip bulb enzyme in the crude extract is about five times that of the aryl acylamidase in the crude rice leaf extract.[8]

TABLE 1. DISTRIBUTION OF AN ARYL ACYLAMIDASE IN TULIP WHICH HYDROLYZES PROPANIL

Tulip tissue*	Units†/g (fresh wt.)	Total units	Protein (mg/ml)	Specific activity‡
Bulbs	366·8	7336	1·6	53·6
Leaves	8·1	161	4·8	0·4
Stems	6·9	138	1·2	1·5

* Mature tulip plants (*Tulipa gesneriana* cv. Darwin) were grown under greenhouse conditions. The plants were harvested in the flowering stage and 20 g fresh wt. of each tissue was used in the preparation of crude homogenates. Assays were carried out at 25° with freshly prepared, cell-free extracts.

† An enzyme unit is defined here as the amount of enzyme required for the hydrolysis of 1 nmole of propanil per hr according to the standard assay conditions.

‡ Specific activity is defined as the number of enzyme units per mg of protein.

Centrifugation of the crude homogenates at 150,000 *g* for 90 min resulted in no loss of enzymatic activity from the crude tulip bulb supernatant. In contrast to both the rice[8] and rat liver[4] aryl acylamidases, the tulip enzyme does not appear to be associated with particulate cell constituents.

Isolation and Partial Purification of a Tulip Aryl Acylamidase Which Hydrolyzes Propanil

The isolation and partial purification of an aryl acylamidase from tulip bulbs was achieved in the same manner as that for a deacetylase from tulip.[9] An acetone powder was prepared from mature tulip bulbs and extracted with phosphate buffer. The extract was subjected to ammonium sulfate precipitation. The enzymatically active pellet was further purified on a Sephadex G-75 column. This 2·8-fold purified Sephadex G-75 fraction was used in all studies presented here.

Stability of Tulip Enzyme at Various Temperatures

The propanil-hydrolyzing enzyme when exposed to a range of temperatures (Table 2) for 5 min prior to assay was stable to about 55° but lost activity rapidly above this temperature. Similar results were obtained when 2′-nitroacetanilide was used as substrate. The acylamidase in liver homogenates was destroyed by a 15 min exposure at 60°.[4]

[9] R. E. HOAGLAND and G. GRAF, *Enzymologia*, in press (1971).

TABLE 2. EFFECT OF TEMPERATURE ON TULIP ARYL ACYLAMIDASE STABILITY

Temperature (°C)	Hydrolysis product (nmole/hr)	Relative activity (%)
25	26·0	100
38	24·1	92
45	23·3	89
52	23·2	89
61	20·0	77
65	8·5	33
74	3·5	13

The complete reaction mixture consisted of 150 μmoles phosphate buffer, pH 7·5, 0·8 μmole substrate, 175 μg Sephadex G-75 enzyme, and de-ionized water to a total vol. of 3·0 ml. The enzyme was exposed to each temp. for 5 min prior to addition to the reaction mixture. The reaction was then incubated at 25° for 3 hr. Termination of the reaction was achieved by the addition of 2·0 ml of acid solution (8 vol. of 1 N HCl and 1 vol. HOAc). Controls consisted of adding the acid mixture prior to addition of the enzyme. Quantitative determination of the 3,4-dichloroaniline produced was then carried out as described in the Experimental.

Effect of Assay Temperature on the Enzyme Activity

The enzyme was assayed at various temperatures (Table 3), the temperature optimum for the hydrolysis of 3′,4′-dichloropropionanilide being 53°. Little hydrolysis was noted above an assay temp. of 65°. When 2′-nitroacetanilide was used as substrate, similar results were obtained.[9] The data from assaying the enzyme at various temperatures when plotted in the Arrhenius form resulted in an apparent activation energy of 10·3 kcal/mole for the hydrolysis of 3′,4′-dichloropropionanilide.

TABLE 3. EFFECT OF ASSAY TEMPERATURE ON THE ENZYME ACTIVITY

Temperature (°C)	Hydrolysis product (nmole/hr)	Relative activity (%)
25	26·1	100
35	47·0	180
46	82·2	315
50	103·5	397
53	120·3	461
61	83·7	321
67	18·2	70
74	7·1	27

The complete reaction mixture consisted of 150 μmoles phosphate buffer, pH 7·5, 0·8 μmole substrate, 175 μg Sephadex G-75 enzyme, and deionized water to a total vol. of 3·0 ml. The reaction was incubated at each temp. for 60 min and then terminated by acid addition. Controls were made by addition of the acid mixture prior to enzyme addition. The 3,4-dichloroaniline produced was then quantitatively determined.

Effect of pH on Tulip Aryl Acylamidase Activity

The enzyme is active in a wide range of pH values (Table 4). The optimum was rather broad, ranging between 6·8 and 7·8. This optimum pH is similar to that of an aryl acylamidase from rice.[8] The tulip bulb enzyme, when assayed with 2′-nitroacetanilide, also revealed a similar pH effect. The acylamidase activity in liver homogenates was highest at a pH of 8·7 when propanil was used as substrate.[4] Nimmo-Smith[10] and Mahadevan and Tappel[11] have shown that aryl acylamidases from chicken and rat kidney preparations also possess pH optima in this range.

Enzyme Stability

Crude extracts stored at 4° retained activity for 3–4 days after extraction. Acetone powder preparations from tulip bulbs could be stored desiccated at 4° for several months with little activity loss. The enzyme from the Sephadex G-75 eluate was stable for several days at 4°. Activity could also be retained for several weeks by freezing the enzyme-buffer solution at −20°.

TABLE 4. EFFECT OF pH ON TULIP ARYL ACYLAMIDASE ACTIVITY

pH	Hydrolysis product (nmole/hr)	Relative activity (%)
5·6	10·3	40
5·7	17·3	66
6·1	21·5	82
6·9	26·1	100
7·1	25·7	99
7·6	25·6	98
8·0	25·4	97
8·2	25·2	97
8·5	24·7	95
9·0	23·8	91

The complete reaction mixture consisted of 150 μmoles phosphate buffer, adjusted to the desired pH, 0·8 μmole substrate, 175 μg Sephadex G-75 enzyme, and de-ionized water to a total vol. of 3·0 ml. The reaction was incubated at 25° for 3 hr and terminated by addition of the acid mixture. Controls were made by adding the acid mixture prior to adding the enzyme. The 3,4-dichloroaniline produced was then quantitatively determined.

Inhibition Studies on Tulip Aryl Acylamidase

Several metal ions and sulfhydryl reagents were incubated with the enzyme to see if they influenced hydrolytic activity. These results are shown in Table 5 with both 3′,4′-dichloropropionanilide and 2′-nitroacetanilide as substrates. The inhibition in both cases was essentially the same. The sulfhydryl reagents *N*-ethylmaleimide, *o*-iodosobenzoate and iodoacetate gave only minimal inhibition, indicating a lack of sensitive-SH groups important for enzyme activity. In contrast, the aryl acylamidase from rice was highly inhibited by these three sulfhydryl reagents.

[10] R. H. NIMMO-SMITH, *Biochem. J.* **75,** 284 (1960).
[11] S. MAHADEVAN and A. L. TAPPEL, *J. Biol. Chem.* **242,** 2369 (1967).

TABLE 5. INHIBITION STUDIES ON TULIP ARYL ACYLAMIDASE

Inhibitor	Concentration (mM)	Inhibition (%) 3′,4′-Dichloropropionanilide	2′-Nitroacetanilide
$HgCl_2$	0·25	100	100
$CuSO_4$	0·25	16	19
N-Ethylmaleimide	0·50	9	10
o-Iodosobenzoate	0·25	5	7
Iodoacetate	0·50	2	4
$CoCl_2$	0·50	1	3
$FeCl_3$	0·50	0	0

The complete reaction mixture consisted of 150 μmoles phosphate buffer, pH 7·5, inhibitor, 0·8 μmole of substrate, 175 μg Sephadex G-75 enzyme, and de-ionized water to a total vol. of 3·0 ml. The inhibitors were incubated with the enzyme, buffer and water for 10 min prior to substrate addition. The reaction was incubated at 25° for 3 hr and then assayed according to the standard procedure.

Substrate Specificity

The tulip aryl acylamidase was tested with a variety of compounds (Tables 6 and 7) to determine the substrate specificity of the enzyme. The enzyme possessed a broad specificity on mono- and dichlorosubstituted propionanilides (Table 6). The same specific activity was noted with 3′,4′-dichloropropionanilide, 2′,4′-dichloropropionanilide and 4′-chloropropionanilide as substrates. The rice aryl acylamidase also exhibited a broad enzyme specificity for these compounds.[8]

A group of alkyl analogs of 3,4-dichloroanilide were also tested in substrate specificity studies (Table 7), the preferred substrate being 3′,4′-dichloropropionanilide. When 3,4-dichloroacetanilide was used as substrate the hydrolysis rate was reduced to 50%. Increasing the carbon chain length of the substrate also resulted in lower hydrolysis. The tulip enzyme also hydrolyzed of 2′-nitroacetanilide and 4′-nitroacetanilide.[9] When compared to propanil as 100%, the relative hydrolysis of the former compound was 79% and the latter compound was 59%.

The tulip enzyme showed no hydrolytic activity on the following compounds: 3-(4-chlorophenyl)-1,1-dimethylurea (monuron), 3-phenyl-1,1-dimethylurea (fenuron), isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) and isopropyl *N*-phenylcarbamate (IPC). This is in contrast to the broad substrate specificity of an enzyme isolated from a soil microorganism which hydrolyzed CIPC, IPC, 3′,4′-dichloropropionanilide and 3,4-dichloroacetanilide.

The partially purified G-75 fraction and the tulip deacetylase[9] exhibit closely similar inhibition characteristics, heat stability, pH optimum and substrate specificity. This suggests that the tulip deacetylase and the tulip enzyme which hydrolyzes propanil are the same enzyme.

Kinetic Studies

A double reciprocal plot of initial velocities at several concentrations of 3′,4′-dichloropropionanilide yielded a straight line. The kinetic data were processed on an IBM 360/50

TABLE 6. SUBSTRATE SPECIFICITY OF TULIP ARYL ACYLAMIDASE ON VARIOUS CHLORINE RING-SUBSTITUTED PROPIONANILIDES

Substrate	Hydrolysis product (nmole/hr)	Relative activity (%)
3′,4′-Dichloropropionanilide	26·2	100
2′,4′-Dichloropropionanilide	26·1	100
4′-Chloropropionanilide	26·0	99
2′-Chloropropionanilide	17·3	66
2′,3′-Dichloropropionanilide	10·7	41
3′-Chloropropionanilide	7·1	27
2′,6′-Dichloropropionanilide	0·5	2

The complete reaction mixture consisted of 150 μmoles phosphate buffer, pH 7·5, 0·8 μmole substrate, 175 μg Sephadex G-75 enzyme, and de-ionized water to a total vol. of 3·0 ml. The reaction was incubated at 25° for 3 hr. Appropriate controls were made by adding the acid mixture prior to the addition of the enzyme. The amount of each chloroaniline produced was determined using standard curves for each product as described in Experimental.

computer using the Fortran program of Cleland.[12,13] The apparent K_m thus determined was $2{\cdot}50 \times 10^{-3}$ M. This K_m value is very similar to that calculated for the aryl acylamidase from rice.[8]

TABLE 7. SUBSTRATE SPECIFICITY OF TULIP ARYL ACYLAMIDASE ON SEVERAL 3,4-DICHLOROANILIDE ANALOGS

Substrate	Hydrolysis product (nmoles/hr)	Relative activity (%)
3′,4′-Dichloropropionanilide	26·2	100
3,4-Dichloroacetanilide	12·8	49
3′,4′-Dichlorobutyranilide	4·7	18
3′,4′-Dichlorovaleranilide	0·79	3
3′,4′-Dichloro-2-methacrylanilide	0·52	2

The complete reaction mixture consisted of 150 μmoles phosphate buffer, pH 7·5, 0·8 μmole substrate, 175 μg Sephadex G-75 enzyme, and de-ionized water to a total vol. of 3·0 ml. The reaction was incubated at 25° for 3 hr and terminated by acid addition. Controls were made by the addition of the acid mixture prior to enzyme addition. The 3,4-dichloroaniline produced was quantitatively determined.

EXPERIMENTAL

Enzyme isolation and purification. Mature garden tulips (*Tulipa gesneriana* cv. Darwin) in the flowering stage were used as sources of plant tissue for the preparation of crude homogenates. 20 g (fresh wt.) each of tulip bulbs, leaves and stems were washed and blotted with paper towels to remove excess water. Each tissue sample was cut into small pieces and homogenized for 90 sec at high speed in an electric blender. The homogenate was then squeezed through several layers of cheesecloth and the filtrate was centrifuged at 0° at

[12] W. W. CLELAND, *Nature, Lond.* **198,** 463 (1963).
[13] W. W. CLELAND, in *Advances in Enzymology* (edited by F. F. NORD), Vol. 29, p. 1, Interscience, New York (1967).

10,000 *g* for 30 min. The pellet was discarded and the supernatant was designated as the crude extract and used in the enzyme distribution and sedimentation studies. Preparation of the Sephadex G-75 enzyme fraction is described in detail elsewhere.[9]

Enzyme assays. The standard reaction mixture contained 0·8 μmole of substrate, 150 μmoles of potassium phosphate buffer, pH 7·5, enzyme and de-ionized water in a total of 3·0 ml. The reaction was initiated by the addition of the enzyme and incubated at 25°. To stop the reaction 2·0 ml of an acid solution (8 vol. 1 N HCl and 1 vol. HOAc) was added. Controls consisted of addition of the enzyme after the 2·0 ml of acid solution had been mixed with the buffer and substrate. The enzyme purification, temperature effects, pH optimum, inhibitor and kinetic studies were all completed with 3′,4′-dichloropropionanilide as substrate. The substrate specificity studies, as well as the standard reaction, were based on the rate of aniline formation. A modified Riden and Hopkins[14] procedure was used to determine the amount of aniline produced. Standard curves were made for each of the chloroanilines used. The chloroanilines were purified by recrystallization and sublimation or preparative TLC before use. After the enzyme reactions were stopped by addition of the acid mixture, the precipitated protein was removed by centrifugation and a 3·0-ml aliquot of the supernatant was analyzed. Initially, 0·5 ml of freshly prepared 1% $NaNO_2$ was added to the aliquot with immediate mixing. After 10 min, 1·0 ml of 10% sulfamic acid, followed by 1·0 ml MeOH, was added and mixed to remove excess nitrite. This mixture was allowed to stand 10 min. The diazonium salt of aniline thus formed was then coupled by addition of 0·5 ml of 1% *N*-naphthylethylenediamine dihydrochloride. This solution was allowed to stand for 15 min, after which the absorbance at 560 nm was determined. The extent of hydrolysis was then determined by comparison of the change in absorbance to a standard curve.

Assays with 2′-nitroacetanilide and 4′-nitroacetanilide as substrates are described elsewhere.[9] An enzyme unit is described here as the amount of enzyme required to hydrolyze 1 nmole of acyl anilide per hr under the assay conditions. Protein was determined by the method of Lowry *et al.*[15] with bovine serum albumin as the standard protein.

Reagents. High purity ring substituted and alkyl substituted anilides used as substrates were synthesized by the Huffman and Allen procedure.[16] Sephadex G-75 was purchased from Pharmacia Fine Chemicals.

High purity pesticides were provided as follows: 3′,4′-dichloropropionanilide (propanil) from Rohm and Haas Company, Philadelphia, Pennsylvania; *N*-(3,4-dichlorophenyl) acrylamide (dicryl) from Niagara Chemical Division, FMC Corporation, Middleport, New York; 3-(4-chlorophenyl)-1,1-dimethylurea (monuron) and 3-phenyl-1, 1-dimethlurea (fenuron) from E. 1. duPont de Nemours & Company Wilmington, Delaware; isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) and isopropyl *N*-phenylcarbamate (IPC) from Pittsburgh Plate Glass Company, Pittsburgh, Pennsylvania.

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[14] J. R. Riden and T. R. Hopkins, *J. Agric. Food Chem.* **9**, 47 (1961).
[15] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
[16] C. W. Huffman and S. E. Allen, *J. Agric. Food Chem.* **8**, 298 (1960).

Key Word Index—Tulipa gesneriana; Liliaceae; herbicide metabolism; aryl acylamidase; 3′,4′-dichloropropionanilide hydrolysis.