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S-Acyl Glutathione Thioesterase of Plant Tissue[1]

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ABSTRACT

The enzymic hydrolysis of S-acyl glutathione has been observed in extracts of various plant tissues. The richest source found in this study was avocado mesocarp. No enzymic activity was observed with acetyl coenzyme A or acetylthiocholine as substrates. Hydrolytic activity was essentially constant in the pH range 7 to 9. There was a break in the activation energy plot at 25 C with activation energy above that point being 6800 calories and 2600 calories below it. The Michaelis constants for S-acetyl, S-propionyl, and S-butyryl glutathione were 200, 80, and 15 μM, respectively. The data are not consistent with the hypothesis that variation in the phytotoxicity of peroxyacyl nitrates is a function of the ability of the plant to hydrolyze glutathione thioesters.

Although the toxic properties of an uncharacterized compound in polluted urban air were recognized in 1956 (15), the chemical structure of peroxyacetyl nitrate ($CH_3CO \cdot OONO_2$) was not elucidated until 1960 (14). Peroxyacetyl nitrate is an oxidizing component of photochemical air pollution and is responsible for eye irritation in man and severe damage to plants (17). Higher homologs have been synthesized, such as peroxypropionyl nitrate and peroxybutyryl nitrate, and their properties of eye irritation and plant toxicity have been examined (17). Higher homologs are increasingly eye-irritating and increasingly phytotoxic (17). Most lachrymatory of all the homologs is peroxybenzoyl nitrate (6), but the phytotoxicity of this compound has not been determined. The concentrations of peroxyacetyl nitrate and peroxypropionyl nitrate in the Los Angeles atmosphere have been determined and in a given instance, found to be 50 nl/1 and 6 nl/1, respectively (2).

In terms of acute effects, there are large differences in the response of plants and animals to peroxyacetyl nitrate. The median lethal concentration for a 2-hr exposure of mice was 104 to 108 μl/1 (1). The concentrations causing death of plant cells is in the nl/1 range.

In vitro experiments of the reaction of peroxyacyl nitrates with biochemical compounds have shown that sulfhydryl groups are particularly reactive (9, 11, 12). In the case of glutathione, the products included the disulfide and S-acetyl glutathione (10). In considering the biochemical basis for phytotoxicity, we may consider two mechanisms for the differential effects of the homologs: (*a*) the plant has differential capacity to prevent the toxic reaction, (*b*) the plant has differential capacity to counteract the adverse effect of the toxic compound. An example of the first case would be greater uptake by the plant of peroxypropionyl nitrate than peroxyacetyl nitrate. An example of the second case would be differential hydrolysis of the thioesters formed by reaction of peroxyacyl nitrates with glutathione (or other sulfhydryl compounds). It is the second hypothesis that is tested in this paper. According to this hypothesis, the higher peroxyacyl homologs are more toxic because the compounds they form are more resistant to degradation by enzymes in the plant cells. In the course of this work, some enzymological studies of the thioesterase were made and are reported here.

MATERIALS AND METHODS

Plant materials were purchased from local markets. Enzyme preparations were made by homogenizing 150 g of material in twice the volume of 0.25 M sucrose to 10 mM tris-HCl, pH 7.2, at 0 C in a Waring Blendor. The homogenate was squeezed through two layers of cheesecloth, and the filtrate was centrifuged at 17,000*g* for 20 min. The supernatant was decanted and could be measured directly for thioesterase activity. Further manipulations to improve the specific activity are described under "Results."

Substrates. S-Acyl glutathione compounds were made either by the method of Kielley and Bradley (7) or by the method of Wieland and Köppe (18). The former method gave more satisfactory yields. Purity of the compounds was checked by comparing solutions containing known weights of the preparation with known concentrations of succinic anhydride in the hydroxamate reaction (13). Purity was also checked by measuring absorbance at 232 nm using absorption coefficients given by Stadtman (13).

Reaction Mixtures. The thioesterase activity was measured by reacting the thiol product (GSH) with DTNB[2] (4). The reaction was not inhibited by DTNB and so the product formation could be followed continuously by measuring the absorbance increase at 412 nm. Reaction mixtures usually contained 100 μmoles of tris-HCl, pH 7.2, 0.5 μmole of DTNB, 0.2 of μmole substrate, and approximately 0.8 mg of protein in a final reaction volume of 3.0 ml. Reactions were normally started by addition of enzyme. The cuvette was placed either in a Cary 15 spectrophotometer or in a Bausch and Lomb 505 spectrophotometer set at 412 nm and arranged to measure rates. The absorbance change was measured for 5 to 10 min, in order to determine the initial rate. Absorbance changes were converted to molar amounts using the extinction coefficient of 13.6×10^3 for the thiophenolate ion product (4).

Protein Determination. The protein in 0.1 ml of sample

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[2] Abbreviation: DTNB: 5,5′ dithiobis-(2-nitrobenzoic acid).

was precipitated with 9 ml of cold acetone. The precipitate was sedimented by centrifugation, and the supernatant solution was discarded. The precipitate was resuspended in 1.0 ml of water and 2.0 ml of biuret reagent added (5). After color development, the absorbance was read at 550 nm, and protein was determined by comparison with a standard curve prepared with bovine serum albumin. The biuret method was preferred for crude enzyme preparations: for purified preparations the method of Lowry *et al.* (8) was used.

RESULTS

Proportionality with Time and Protein Concentration. The thioesterase reaction catalyzed by the enzyme from spinach leaf, deviates only slightly from linearity over a period of 60 min (Fig. 1a). The reaction rate is proportional to protein concentration within the range used in the experiments reported in this paper (Fig. 1b).

Effects of pH. There was some tendency for nonenzymic hydrolysis of S-acetyl glutathione at alkaline pH levels. When the nonenzymic rate was subtracted, it was apparent that the enzymic activity was essentially constant in the range 7 to 9. The pH of 7.2 was chosen (at the lower end of the tris buffering range) because corrections for nonenzymic hydrolysis were not necessary at that pH. In the case of S-propionyl glutathione, nonenzymic hydrolysis was not observed, and in this case, the effect of pH could be observed directly. The enzymic activity was essentially constant in the range of pH 7 to 9 (Fig. 2).

Effect of Temperature. In the 5-min reaction period, the enzymic hydrolysis of S-acetyl glutathione was stimulated by raising the temperature as high as 60 C (Fig. 3a). When these data were used to obtain an Arrhenius plot, it was noticeable that there was a break at 25 C (Fig. 3b). Calculations of activation energy indicate that above 25 C it was 6800 calories and below 25 C it was 2600 calories. Heating the enzyme in a boiling water bath for 5 min inactivated the enzyme completely, but an indication of the heat stability is that the enhancement of rate caused by raising the temperature to 60 C is maintained for at least 30 min.

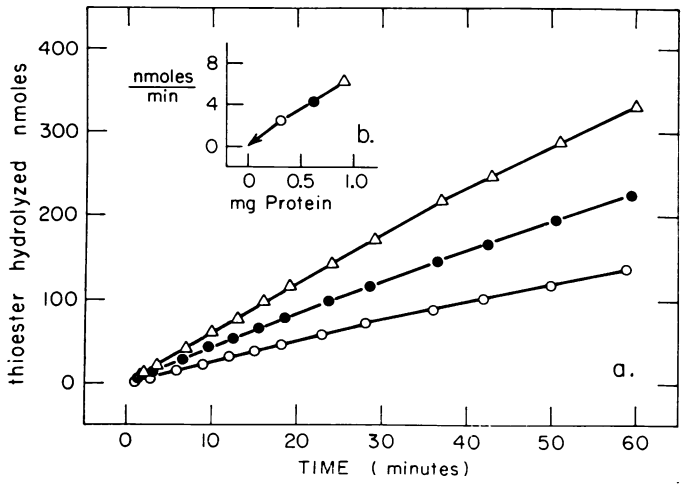

FIG. 1. Time course and protein concentration. Reaction mixtures contained 100 μmoles of tris-HCl pH 7.2, 2 μmoles of S-acetyl glutathione, 0.5 μmoles of DTNB, and various amounts of enzyme in a reaction volume of 3.0 ml. The enzyme was prepared from spinach leaves by taking a 17,000*g* supernatant fraction and making it 55% saturated with $(NH_4)_2SO_4$. The protein precipitate was centrifuged down and redissolved in 0.1 M tris-HCl pH 7.2. Protein concentration was 15.2 mg/ml. The assay was made discontinuously, readings at 412 nm being taken in a Beckman DU spectrophotometer at 22 C.

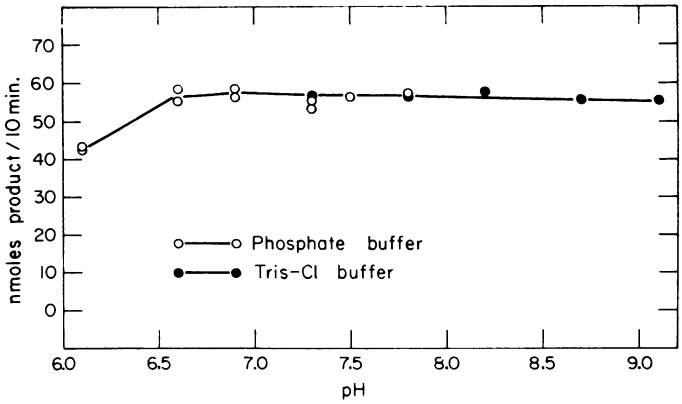

FIG. 2. Effect of pH on the hydrolysis of S-propionyl glutathione. Reaction mixtures contained 100 μmoles of buffer, 0.5 μmole of S-propionyl glutathione, 0.25 μmole of DTNB, and 0.05 ml of spinach leaf enzyme in a final volume of 3.0 ml.

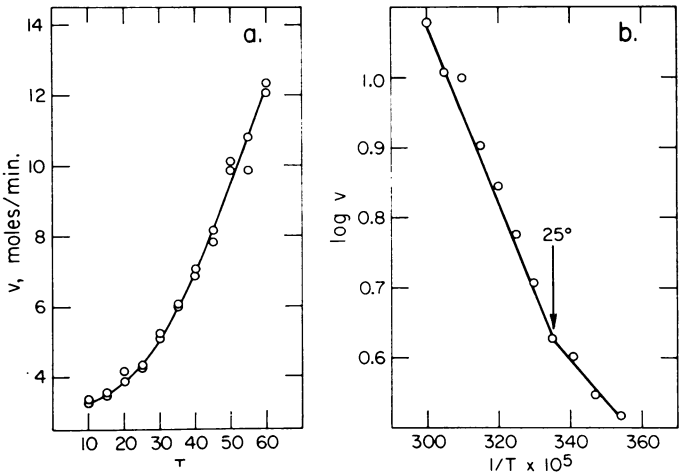

FIG. 3. Effect of temperature. Reaction mixtures were as described in the legend to Fig. 1. Protein in each reaction vessel was 0.12 mg. The enzyme was the 17,000*g* supernatant from an avocado mesocarp homogenate. Reactions were started by additions of enzyme and rates recorded for 5 min with a Cary 15 spectrophotometer. Rates in nmoles/min were calculated from the slopes of the recorder traces. All assays were made in duplicate. a: Temperature *vs.* velocity; b: Arrhenius plot of the data in a. Specific activity at 30 C = 43 nmoles/min·mg protein.

Substrate Range. The activity of the enzyme for spinach leaf was tested with the acetyl-, propionyl-, and butyryl-thioesters of glutathione and also with acetyl coenzyme A and acetyl thiocholine. The former three substrates were readily attacked, but there was no observable hydrolysis of the latter two.

Distribution in Plants. Table I shows the activity of homogenates of various plant tissues which had simply been clarified by centrifugation at 17,000*g*. The avocado fruit was found to have high activity. Spinach leaf was preferred as the source of enzyme in subsequent experiments because this study was designed to test the biochemical basis for toxicity which is most obvious in leaf tissue.

Purification of the Enzyme. Although the purification of the enzyme was not a requirement for the present work, various purification steps were used at different times. Ammonium sulfate fractionation did not give good recoveries of enzymic

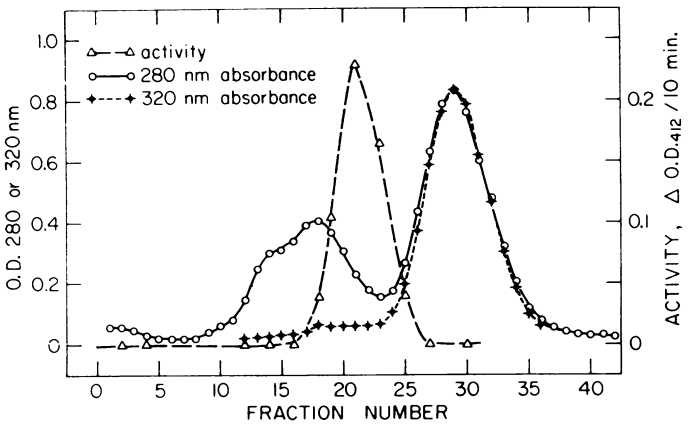

FIG. 4. Chromatography of thioesterase on Sephadex G-200.

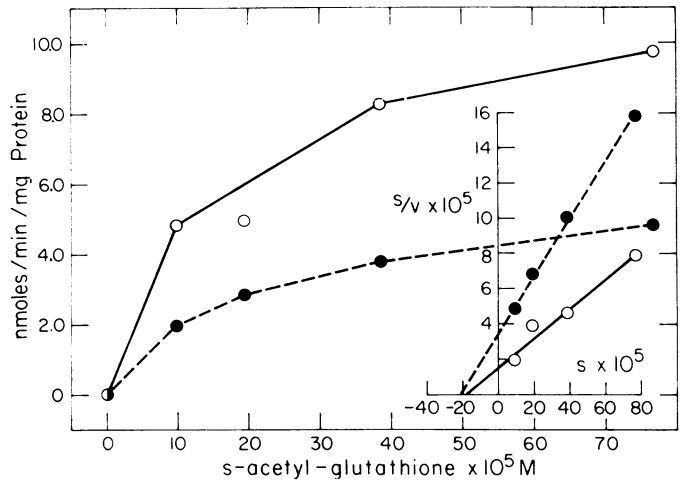

FIG. 5. Kinetic analysis of S-acetyl glutathione hydrolysis. Reaction mixtures were as described in the legend to Fig. 1. Protein in each reaction vessel was 0.47 mg. The enzyme was the 17,000*g* supernatant of a spinach leaf homogenate. Varying concentrations of S-acetyl glutathione were added. Rates were recorded continuously with a Cary 15 spectrophotometer and recalculated as nmoles/min·mg protein. Absorbance changes in the absence of enzyme were recorded before the enzyme was added and the nonenzymic rates subtracted if necessary. Data from the freshly prepared enzyme (○) and enzyme stored frozen for 1 day (●) are presented. The inset shows the data plotted by the method recommended in Fig. 3. Purity of S-acetyl glutathione was 96%.

activity, but the active fractions were more stable than the original homogenate. No purification could be obtained by heat treatment or pH precipitation. Ammonium sulfate fractions could be further purified by chromatography on Sephadex G-200 or on diethylaminoethyl cellulose. Treatment of the homogenate with cold acetone to bring the acetone to 40% caused the formation of a protein precipitate which was inactive. The acetone-water supernatant contained all the enzymic activity and could be assayed directly. Raising the acetone concentration higher than 40% yielded inactive precipitate and supernatant fractions. Further manipulations of the 40% acetone supernatant have yielded preparations of improved specific activity. A purification procedure using the above steps improved the specific activity of a preparation from spinach leaves 20-fold. The Sephadex G-200 step of this procedure is shown in Figure 4. This was the final step and the specific activity was 144 units/mg protein.

Determination of Kinetic Parameters. The effect of changing the substrate concentration on the reaction velocity is shown in Figures 5, 6, and 7 for S-acetyl glutathione, S-propionyl glutathione, and S-butyryl glutathione, respectively. All substrates were run the same day with the same enzyme preparation. The runs were repeated the succeeding day with the same enzyme preparation which had been frozen overnight. This procedure provided confirmation of the *Km* value obtained the 1st day, and measured the inactivation of the activity using the same substrates. Since the inactivation was the same for all three substrates, it seems likely that all three reactions are catalyzed by the same enzyme.

DISCUSSION

We are unaware of previous reports of the thioesterase activity in plants described in this paper. Even in animal tissues, the descriptions are not numerous (7, 16). In both animals and plants, a function cannot be attributed to the enzyme, so one

Table I. *Distribution of Thioesterase*

Plant materials were homogenized in a Waring Blendor as described under "Materials and Methods." The supernatants from the 17,000*g* centrifugation were used as the enzyme. In the case of the avocado, the fraction used was that between the pellet and the floating fat layer. Many determinations of thioesterase activity in avocado and spinach were made. Averages of several preparations are given. Other figures come from duplicate determinations of a single enzyme preparation. The substrate in all cases was S-acetyl glutathione. The amount of enzyme causing the hydrolysis of 1 nmole S-acetyl glutathione/min is defined as one unit.

Plant Tissue	Specific Activity	Protein/reaction
	nmloes/min·mg protein	*mg*
Avocado mesocarp	92.3	0.60
Cabbage leaf	14.5	0.25
Spinach leaf	9.1	0.20
Cucumber fruit	6.1	0.15
Cauliflower inflorescence	6.0	0.24
Potato tuber	4.3	0.36
Bell pepper fruit	2.8	0.24
Celery stalk	0	0.10
Tomato fruit	0	0.15

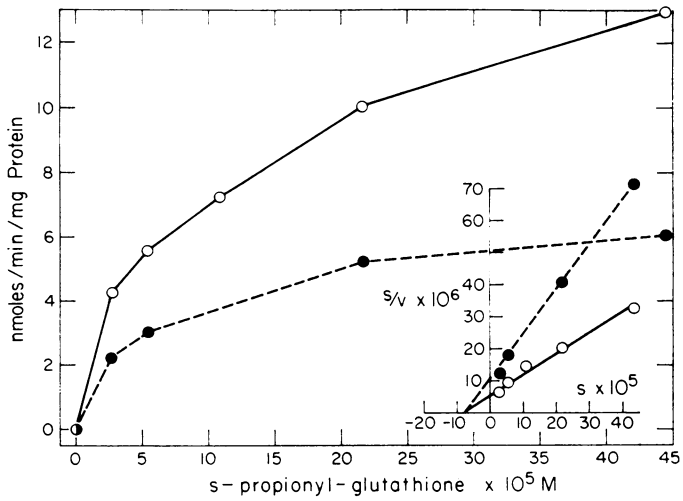

FIG. 6. Kinetic analysis of S-proprionyl glutathione hydrolysis. Procedure as described in the legend to Fig. 3. Purity of S-propionyl glutathione was 81%.

suspects that a facet of some other enzymic activity is being measured. It has been suggested that the thioesterase hydrolyzing S-acetyl glutathione is possibly the same enzyme as glyoxylase II, which hydrolyzes S-lactoyl glutathione, but this suggestion was nullified when Kielley and Bradley were able to resolve the two thioesterase activities (7). It would not have helped our understanding of the enzymes role much if they had been the same because glyoxylase II also has no known function. Kielley and Bradley (7) also considered the possibility that the thioesterase in mouse liver was a simple esterase; however, this possibility was made unlikely by differential purification of the two activities.

We have not ascertained whether the thioesterase activity in plants can be ascribed to some enzyme which fits more reasonably into schemes of metabolism. While investigating this problem, we found high activity of glyoxylase I, but no direct measurements of glyoxylase II were made. As far as the properties of the thioesterase are concerned, the pH curve for the enzyme from plants (Fig. 2) is very similar to that from brain (16). Strecker *et al.* (16) reported a K*m* for S-acetyl glutathione of 1 mM for this substrate; whereas the value reported in this paper is 0.2 mM (Table II). Acetyl CoA was not a substrate for the enzyme described in animal tissues (7, 16), nor is it a substrate for the plant enzyme.

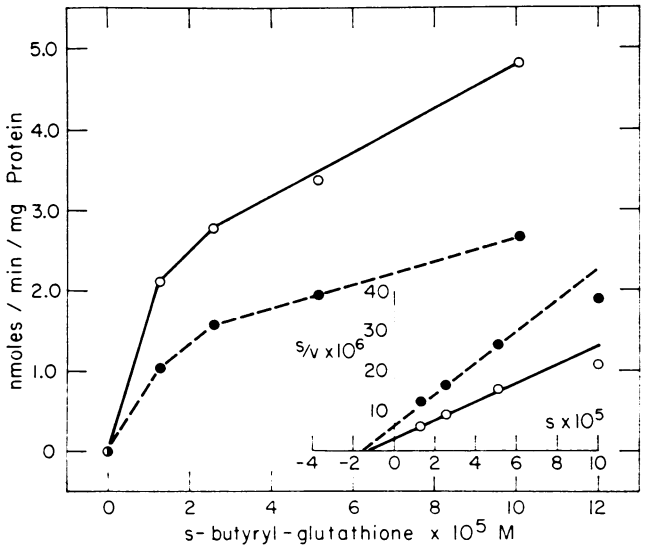

FIG. 7. Kinetic analysis of S-butyryl glutathione hydrolysis. Procedure as described in the legend to Fig. 3. Purity of S-butyryl glutathione was 77%.

Table II. *Kinetic Parameters for Thioesterase*

Data are compiled from Figs. 5, 6, and 7.

Compound	K*m*	V_{max}
	μM	*nmoles/min·mg protein*
S-acetylglutathione	200	13
S-propionylglutathione	80	14.5
S-butyrylglutathione	15	5

One of the purposes for starting the work described in this paper was to determine the reason for the order of plant toxicity peroxybutyryl nitrate > peroxypropionyl nitrate > peroxyacetyl nitrate (17). It has already been reported that peroxyacetyl nitrate acylates GSH (10) and the lowered level of GSH or some other thiol could be the initial reaction which leads to expression of symptoms. According to this hypothesis, S-acyl derivatives of thiols should be more resistant to hydrolysis as the acyl group increases in size. The data for derivatives of glutathione (Table II) do not support the hypothesis. We are in the process of testing alternative hypotheses, such as differential uptake of the three compounds by plant leaves.

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