OXIDATION OF INDOLEACETIC ACID BY QUACKGRASS RHIZOMES[1, 2]

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The enzymatic oxidation of 3-indoleacetic acid (IAA) which was first described in detail by Tang and Bonner (16, 17), has been observed in several types of plants, e.g., fungi (13), ferns (14), monocotyledonous plants (20), and dicotyledonous plants (16, 17, 19). Although differences have been observed in regard to the enzyme's requirement for a phenolic cofactor, and in the pH optimum, the requirement for manganese seems to be general.

Interest in the IAA oxidase of quackgrass rhizomes arose from the finding that the rhizomes showed a seasonal variation in bud activity (5). The hypothesis that the bud activity of quackgrass rhizomes may reflect the concentration of IAA in the rhizomes, and that this concentration may be controlled by IAA oxidase was considered. Others have presented evidence both for (4) and against (2) the participation of IAA oxidase in the control of IAA concentration under physiological conditions. In the course of the present study, some characteristics of the IAA oxidase of quackgrass rhizomes have been compared with the IAA oxidase from other sources. The relationship between total nitrogen of the rhizome tissue, IAA oxidase activity, and the protein content of the enzyme extracts is discussed.

MATERIALS AND METHODS

Rhizomes were obtained from an undisturbed, heavily infested quackgrass sod at weekly intervals during the growing season beginning March 27, 1957. The rhizomes were cut into 20 mm sections, each having 1 bud. These sections were quick frozen in liquid air and lyophilized. The dried sections were ground to 60 mesh size with a Wiley mill, and the resulting powder was stored in a desiccator over phosphorus pentoxide at −15° C until needed.

The enzyme preparations for oxidizing IAA were obtained from lyophilized quackgrass rhizome material by extracting 2 g of the stored powder with 20 ml of distilled water at 5° C for 24 hours. The mixture was centrifuged at 9000 × G for 30 minutes and the supernatant decanted. The residue then was extracted with 10 ml of water for 12 hours; the mixture was centrifuged and the supernatant decanted. The residue finally was washed with 3 ml of water. The combined extracts were dialyzed either against running tap water at 10° C for 40 hours and then distilled water at 5° C for 10 hours, or against several changes of distilled water at 5° C for 48 hours. The dialyzed extracts were made up to a standard volume of 40 ml.

Enzyme preparations were obtained from fresh quackgrass rhizomes by grinding the rhizomes with ice in a Nixtamal mill. The milled material was pressed through cheesecloth, and the collected liquid was centrifuged at 3000 × G for 30 minutes. Solid ammonium sulfate was added to the decanted supernatant to make the solution saturated. The precipitate obtained was redissolved in distilled water, and solid ammonium sulfate was added to this solution to make it 80 % saturated. The precipitate obtained was dissolved in distilled water and dialyzed for 24 hours against running tap water at 10° C and finally against distilled water at 5° C. This preparation was centrifuged, and the clear supernatant was used as the enzyme solution.

Peroxidase activity was measured by following the formation of purpurogallin colorimetrically at 430 mμ with a Bausch and Lomb Spectronic 20 colorimeter. The reaction mixture, in a 100 × 12 mm test tube, consisted of 0.5 ml of 0.1 M phosphate buffer at pH 6.0, 0.5 ml 0.01 M pyrogallol, 0.1 ml of 0.15 % H_2O_2, 0.5 ml of enzyme solution, and water to make a total volume of 3.0 ml. The reaction was started by the addition of the enzyme. Readings were taken at 10 second intervals and the initial rate calculated from the readings of the 1st minute.

The oxidation of manganese was measured by following the formation of manganipyrophosphate spectrophotometrically. Manganipyrophosphate has an absorption peak at 258 mμ (7). Measurements were made in a 1 cm cuvette with a Beckman DU spectrophotometer at 258 mμ. The reaction mixture consisted of 0.5 ml of 0.1 M pyrophosphate at pH 7.0, 0.5 ml of 0.01 M $MnCl_2$, 0.5 ml of 0.001 M resorcinol, 0.1 ml of 0.15 % H_2O_2, 1.0 ml of enzyme solution, and water to make a total volume of 3.5 ml. The reaction was started by addition of the enzyme. Readings were taken at 30 second intervals, and the initial rate was calculated from the readings of the 1st 2 minutes.

The IAA oxidase activity was determined by measuring oxygen uptake at 30° C in a Warburg respirometer (18). The volume of the reaction mixture was 3.0 ml; 0.15 ml of 20 % KOH and a filter paper wick were present in the center well. The reaction was started by the addition of either the enzyme or IAA from the side arm.

The protein content of the enzyme solutions was determined by the method of Lowry et al (8). Reducing sugars were determined by the method of Park and Johnson (10). Total nitrogen content of the powdered, lyophilized rhizome samples was determined by the Kjeldahl procedure. Horseradish peroxidase (HRP) was obtained from Nutritional Biochemicals Corporation. HRP solutions were made by dissolving 10 mg of the powder in 100 ml distilled water.

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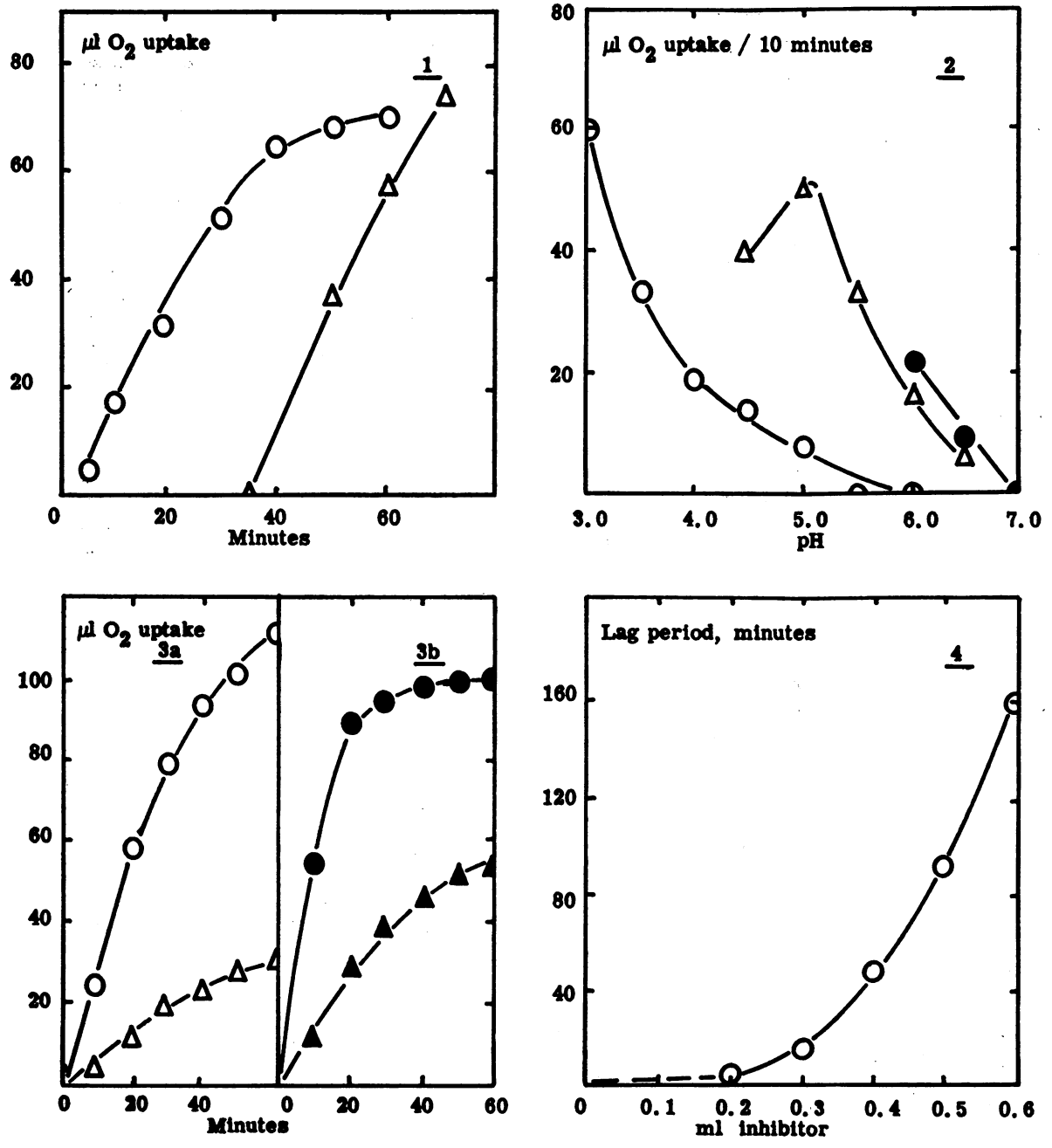

FIG. 1. Effect of dialysis on an IAA oxidizing preparation from quackgrass rhizomes. The reaction mixture contained 0.5 ml 0.1 M phosphate buffer, pH 6.0; 0.3 ml 0.01 M $MnCl_2$; 0.3 ml 0.01 M resorcinol; 1.0 ml 0.01 M IAA; 0.5 ml enzyme solution. △, before dialysis; ○, after dialysis.

FIG. 2. Effect of buffer and pH on IAA oxidation by quackgrass rhizome preparations. The reaction mixture contained 0.5 ml 0.1 M buffer; 0.3 ml 0.01 M $MnCl_2$; 0.2 ml 0.01 M resorcinol; 0.5 ml 0.01 M IAA; 1.0 ml enzyme solution. (The pH range tested with phosphate buffers was limited, because they buffer poorly in the region directly below pH 6.) ○, citrate; △, succinate; ●, phosphate.

FIG. 3. Effect of cofactors on the oxidation of IAA by quackgrass rhizome preparations. The complete reaction mixture for 3 a was the same as in figure 2; phosphate buffer pH 6.0. In 3 b resorcinol was replaced by an equal amount of 2,4-dichlorophenol. △, enzyme + IAA + resorcinol; ○, enzyme + resorcinol + IAA + Mn^{2+}; ▲, enzyme + 2,4-dichlorophenol + IAA; ●, enzyme + 2,4-dichlorophenol + Mn^{2+} + IAA. Oxygen uptake in the absence of phenolic cofactor was negligible.

FIG. 4. Inhibition of the IAA oxidizing activity of HRP by the naturally occurring inhibitor from quackgrass rhizomes. The reaction mixture contained 0.5 ml 0.1 M phosphate buffer, pH 6.0; 0.3 ml 0.01 M $MnCl_2$; 0.2 ml 0.01 M resorcinol; 0.5 ml 0.01 M IAA; 0.2 ml HRP solution; rhizome extract.

Results and Discussion

The presence of an IAA oxidizing enzyme in quackgrass rhizomes was demonstrated readily. Extracts from powdered, lyophilized quackgrass rhizomes were found to contain a dialyzable inhibitor(s) of the enzyme, as shown in figure 1. Subsequently all preparations were dialyzed for at least 48 hours before assay.

The efficiency of the extraction procedure for the lyophilized rhizome powders was tested. Two grams of the powder was extracted for 12 hours with 20 ml distilled water. The supernatant was decanted after centrifuging at 9000 × G for 30 minutes. Five more similar extractions were made with 10 ml portions of water. The 6 samples were dialyzed for 48 hours before assay. The results (table I) show that all of the enzymatic activity was obtained in 3 extractions.

Table I

Enzyme Extraction from Powdered, Lyophilized Quackgrass Rhizomes

Extract	Protein, μg/ml	Mn oxidase, ΔO.D./min.	Peroxidase, ΔO.D./10 sec	IAA oxidase, μL O_2/10 min
1	375	0.060	0.040	7.0
2	220	0.025	0.015	2.5
3	121	0.007	0.007	0
4	56	0	0	0
5	47	0	0	0
6	36	0	0	0

The reaction mixture for the oxidation of IAA contained 1.0 ml of enzyme extract; 0.5 ml 0.1 M phosphate buffer pH 6.0; 0.3 ml 0.01 M $MnCl_2$; 0.2 ml 0.01 M resorcinol; 0.5 ml 0.01 M IAA.

Other assays and analyses were made as described in Materials and Methods.

The effect of pH on the oxidation of IAA by the enzyme preparation from fresh quackgrass rhizomes was tested with 3 buffers. The results with orthophosphate and succinate buffers agreed in the pH range where both were tested. A pH optimum of about 5 is indicated with succinate. With citrate buffer, the apparent pH optimum is much lower (fig 2). Attention has been drawn to the chelation of manganic ions by citrate (6); a pH optimum for IAA oxidase determined with citrate buffer is invalidated by chelation. Appreciable activity in citrate buffer should be observed only at low pH where the undissociated acid and the monoanion, which do not chelate, are the predominant forms. The acid pH optima reported for some IAA oxidase preparations may be attributed to the use of citrate buffer (12). Chelating effects of buffers used may explain some of the differences in pH optima found for IAA oxidase preparations from different sources.

The cofactor requirements of the IAA oxidizing enzyme of quackgrass rhizomes appear to be the same as those for the enzymes found in wheat leaves (9) and in lupine hypocotyls (15). The effect of cofactors on a preparation from fresh rhizomes is shown in figure 3. The cofactors had the same relative effect on enzymes from powdered, lyophilized rhizomes.

The naturally occurring inhibitor(s), which is heat stable, causes a lag period in oxidation of IAA. A crude inhibitor preparation was obtained by boiling 2 g of the powder with 20 ml of distilled water. The supernatant was decanted and the extraction repeated 3 times with 10 ml portions of hot water. The inhibitor content of the combined extracts was assayed by measuring its effect on the oxidation of IAA by a HRP preparation. Figure 4 shows that the lag period induced increases disproportionately (approximately exponentially) as the concentration of inhibitor is increased. The lag period may be interpreted as arising from the inactivation of an essential intermediate by the inhibitor. This could result in the concomitant inactivation of the inhibitor. After the concentration of the inhibitor has been reduced to an innocuous level, the reaction should be free to proceed. A lag period in the oxidation of IAA has been observed with the naturally occurring inhibitor from wheat leaves and with catechol (9). A lag period is also found with agents which chelate manganese, such as citrate and pyrophosphate.

In March 1958, after a minimum of 7 months storage of the powdered, lyophilized quackgrass rhizomes, it was found that even prolonged dialysis of their extracts did not remove all of the naturally occurring inhibitor of IAA oxidase. However, no inhibition of peroxidase and manganese oxidase activities was observed. Ray (11) has discussed the role of manganese in the enzymic oxidation of IAA. According to one theory (9), the oxidation depends on the formation of manganic ions. If this is the case, the inhibition by the naturally occurring inhibitor must take place in the reaction sequence after the formation of manganic ions. The inhibition of the endogenous IAA oxidase from several dialyzed sample extracts is shown in figure 5. Extracts from samples harvested later in the growing season showed longer lag periods even though the powders had been stored for a shorter length of time when the determination was made. The same samples caused a lag period in IAA oxidation as shown in figure 6. After the lag period was overcome, the sum of the activities of the endogenous enzyme and the HRP gave greater rates of oxygen uptake than that observed with HRP alone.

After prolonged dialysis, the extract might be expected to contain polysaccharide in addition to protein. The principal polysaccharide of quackgrass rhizomes has been shown to be a complex fructosan (1), which has been called triticin. Triticin was obtained from extracts showing a non-dialyzable inhibitor by a modification of the method of Arni and Percival (1). One hundred ml of the extract was treated with basic lead acetate and the precipitate removed by filtration. Hydrogen sulfide was bubbled through the filtrate to precipitate the lead, and then air was bubbled through

to remove excess hydrogen sulfide. The lead sulfide was removed by filtration, and the filtrate was concentrated to 10 to 15 ml at 40° C and a pressure of 15 mm Hg. The triticin was precipitated by pouring this solution into 300 ml ethanol, and was recovered by filtration. The triticin was re-precipitated 3 times from water with ethanol. The product was washed with ether and dried in a vacuum desiccator. As previously described (1), the triticin was hygroscopic, and in solution it showed no reducing power. A 2 % solution gave a specific rotation of −42°. After hydrolysis in 0.1 *N* HCl in a 55° C water bath for 30 minutes, the specific rotation was −72°. This value was not changed on further heating.

The purified triticin did not inhibit the oxidation of IAA by HRP, indicating that the polysaccharide itself is not the non-dialyzable inhibitor. Glucose and fructose were also tested and did not inhibit the enzymic oxidation.

It is probable that the inhibitory substances are bound to high molecular weight compounds and so are non-dialyzable. Perhaps fresher material contains an enzyme, lost on aging, which can release the inhibitory substances, and in extracts from aged material, devoid of the enzyme, the inhibitor is not released in a dialyzable form.

A cold water extract of powdered, lyophilized quackgrass rhizomes was hydrolyzed with 0.1 *N* HCl and extracted with several portions of ether. The ether was removed by evaporation and the residue dissolved in water. The ether soluble and water soluble fractions were compared with the unfractionated hydrolyzed sample. Most of the inhibitor remained in the aqueous phase. A similar fractionation was performed with an inhibitor preparation obtained by extraction with hot water, and a similar distribution of the inhibitor was observed. This behavior is comparable to that observed with the naturally occurring inhibitor of wheat leaf IAA oxidase (9).

The protein content of the IAA oxidase preparations paralleled the total nitrogen content of powdered, lyophilized rhizome tissue, indicating that changes in IAA oxidase were merely part of a general change in nitrogenous constituents. Further determinations showed that though the total IAA oxidase activity of the rhizomes changed with the season, the specific activity per unit of nitrogen remained virtually constant during these seasonal changes. The total IAA oxidase activity was directly related to the rates of oxidation measured, since a standard volume of extract was obtained from a standard weight of powder. The reduction in IAA oxidase activity as the season progressed was simultaneous with the increase in inhibitor. If these 2 factors are effective in vivo there will be less IAA oxidation as the season progresses.

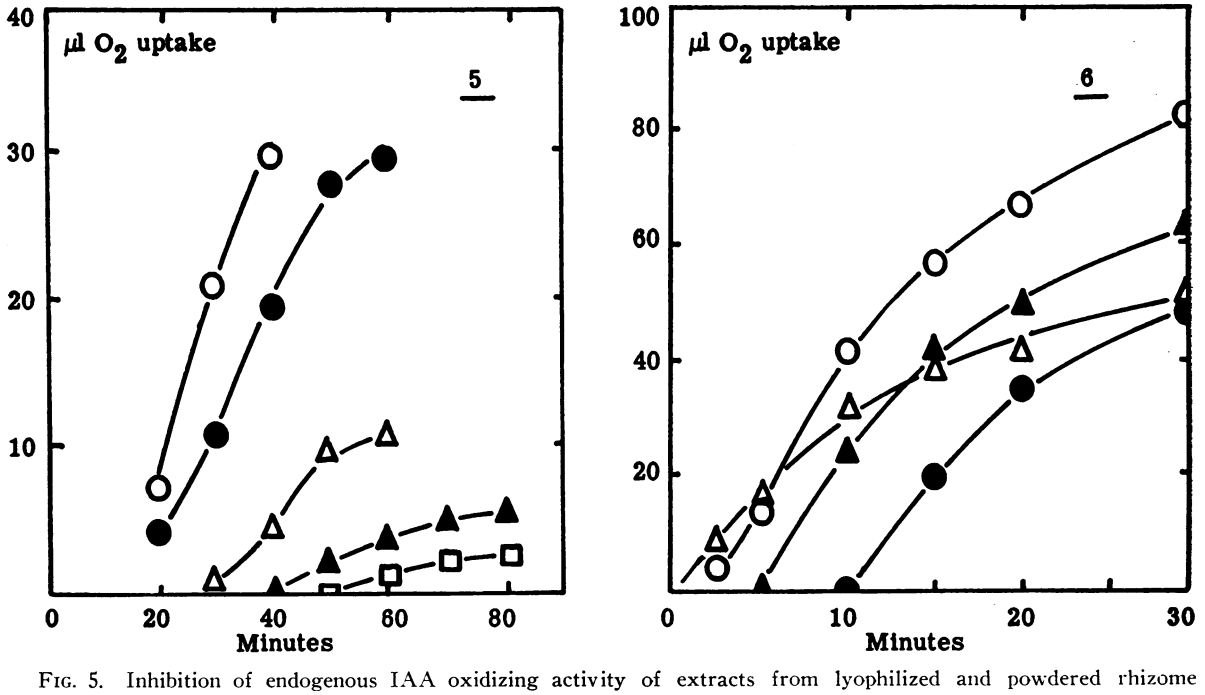

FIG. 5. Inhibition of endogenous IAA oxidizing activity of extracts from lyophilized and powdered rhizome samples by a non-dialyzable inhibitor. Sampling dates: ○, 4–3; ●, 4–24; △, 5–22; ▲, 6–5; □, 8–7. Reaction mixtures as in table I.

FIG. 6. Inhibitory effect on IAA oxidizing activity of HRP of extracts from powdered, lyophilized rhizomes showing non-dialyzable inhibition. △, HRP alone; ○, HRP + extract from sample taken 4-3; ▲, HRP + extract from sample taken 5–22; ●, HRP + extract from sample taken 8–7.

It is interesting that the changes in IAA oxidase, manganese oxidase, and peroxidase correspond. This is a further indication that IAA oxidase activity is a facet of peroxidase activity, as suggested by Galston et al (3) and later by Stutz, (15), and depends on the peroxidatic oxidation of manganese (table II).

TABLE II

ENZYMIC ACTIVITIES OF EXTRACTS FROM POWDERED, LYOPHILIZED QUACKGRASS RHIZOMES

SAMPLING DATE	% TOTAL N IN DRIED SAMPLE	PROTEIN μG/ML	Mn OXIDASE ΔO.D./MIN	PEROXIDASE ΔO.D./10 SEC	IAA OXIDASE μL. O_2/10 MIN	IAA OXIDASE QO_2 (N)
4–3	0.68	465	0.060	0.025	14.0	1,130
4–24	0.58	310	0.040	0.020	10.0	1,210
5–22	0.24	245	0.030	0.010	7.5	1,100
6–5	0.20	205	0 023	0.010	6.0	1,100
8–7	0.37	290	0.028	0.010	7.5	970

SUMMARY

Quackgrass rhizomes contain an IAA oxidase whose characteristics and cofactor requirements, for manganese and a phenolic compound, are similar to those reported for the enzyme from other plant sources. The rhizomes contain an inhibitor(s) which causes a lag period in the oxidation of IAA. The length of the inhibition period increases disproportionately (approximately exponentially) as the concentration of the inhibitor is increased. It was possible to remove the inhibitor from extracts of fresher samples of lyophilized rhizomes by dialysis; extracts from aged samples retained the inhibitor even after prolonged dialysis. The inhibition does not appear to be associated with the complex polysaccharide present in the rhizomes or its monosaccharide components. The inhibitor remained in the aqueous phase when solutions of the inhibitor were extracted with ether.

The total IAA oxidase activity of the quackgrass rhizomes showed a seasonal variation, but the specific activity remained virtually constant during the same period. The data indicate that variations in IAA oxidase activity are closely associated with general changes in the nitrogenous constituents of the rhizome tissue. The decrease in IAA oxidase activity and increase in inhibitor take place at the same time. If these effects are operative in vivo, there will be less IAA oxidation as the season progresses.

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