PRELIMINARY STUDIES OF THE ENZYMES OF GIBBERELLA SAUBINETII[1]

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INTRODUCTION

Gibberella saubinetii (Mont.) Sacc., the fungus causing the commonly known scab disease of maize, oats, wheat, barley, and a few other cereals, has engaged the attention of scientists for some time as a disease of some of the world's most important economic crops. Its physiological properties and characteristics are fairly well established among botanists, but the biochemical properties of the organism are not so well known. Studies on the enzymic activity of the fungus were undertaken in order to give more information concerning some of the chemical changes that occur within the affected host tissues, and possibly to throw some light on other enzymic properties which have hitherto been unknown.

REVIEW OF LITERATURE

It is known from investigations that cereals attacked by this fungus are toxic when eaten by animals. Naumov (*8*)[3] submitted evidence that wheat grains affected by scab acquire toxic properties that are subsequently communicated to the flour and bread. The toxin produced, supposed to be a nitrogenous glucoside, when injected into frogs resulted in death in three or four hours. Naumov's work is corroborated in part by Bennett (*1*). Mundkur and Cochran (*7*) report that hogs fed on an exclusive diet of infected barley developed nausea for food and starved rather than eat it. Guinea pigs also rejected such an exclusive barley diet. Mature chickens, however, when fed with scabby barley showed no symptoms of poisoning. Dickson and others (*2*) found that the water extract from badly scabbed barley produced acute vomiting in pigs. The extract when freed from proteins, polysaccharides, and those nitrogenous substances precipitable with tannic acid was more acute as an emetic for pigs than the original crude extract. The active substances seemingly are associated with the fractions containing glucosides or basic nitrogen compounds. Roche and his coworkers (*9*) have found that pigs, horses, and dogs, as well as man, are very sensitive to the accumulated products in the affected grains and will not tolerate even low percentages of badly scabbed kernels. However, the farm utilization of scabby barley may be economical because it can be fed to cattle, sheep, and poultry without ill effects.

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[2] The writers wish to acknowledge the cooperation of the botany department, particularly Dr. E. B. Mains.
[3] Reference is made by number (italic) to Literature Cited, p. 229.

GROWTH OF THE SCAB FUNGUS IN THE LABORATORY

The liquid medium best suited for the cultivation of the scab fungus consisted of a 4 per cent malt extract with the addition of 0.2 per cent nitrate of soda. Various other liquid media of both organic and inorganic natures were tried but did not prove satisfactory for the work. Among them were the media used by Ellenberger (*4*) and Leonian (*6*). The medium used was about at the neutral point at the time of inoculation and became slightly acid (usually about pH 5.5 to 5.8) as the growth of the fungus progressed.

The malt-extract medium was prepared by finely grinding malted barley and extracting with distilled water for an hour or longer. After the solution had stood for the required length of time it was filtered to remove the undissolved material. To the filtrate was then added 0.2 per cent nitrate of soda, and the medium was brought up to volume in a volumetric flask. After sterilizing the medium, it was filtered to remove the flocculent precipitate. Resterilizing for 25 to 30 minutes at 15 pounds pressure left the medium perfectly clear with no precipitate.

This resulting medium was inoculated with mycelial growth from test-tube cultures on potato-dextrose agar. The maximum growth was obtained at a temperature of 25° C. on about 1 cm. depth of medium in Erlenmeyer flasks.

On the tenth day, at which time the maximum growth had been produced and showed the pinkish-red coloration, the matted mass on the surface of the medium was removed and dehydrated with acetone and ether according to the method of Dox (*3*). The desiccated mass was dried at room temperature until the odor of ether was no longer perceptible, and the dry mass was ground thoroughly in a mortar. The resulting pinkish-red product was used in making the enzyme studies. It can be kept several months in a dark place and in an air-tight bottle.

COMPOSITION OF ENZYME MATERIAL

The enzyme material contains, besides the enzymes, all the cell constituents that are insoluble in acetone and ether. The acetone from the first dehydration has a reddish-pink color. The residue left after distilling off the acetone resembles lipoids. The ground, extracted enzyme material contains 0.1115 per cent ash and 6.16 per cent total nitrogen. The aqueous extract gives positive tests for the biuret and Hopkins-Cole reactions.

METHODS

In the experiments about to be described the source of enzyme was the prepared powder, thoroughly ground in a mortar to which had been added a few drops of water, glycerin, and a small quantity of sand. The groundmass was transferred with distilled water to the volumetric flasks containing the appropriate substrate, and the contents of the flask were brought up to volume with distilled water to give the correct percentage concentrations of both the enzyme and substrate. The advantage of this method is that a preparation of uniform activity can be used in a whole series of experiments.

The digestions were carried out at room temperature (about 25° C.). The surface of each digestion mixture was kept covered with toluol which served as an antiseptic. All the digestion tubes and flasks were tightly stoppered to prevent any change in volume by evaporation of the solvent and any loss of toluol. Aliquot portions taken for analysis were carefully measured with a pipette. The time of digestion varied in the different experiments but is stated in each case. Control experiments were made from each digestion mixture, and aliquot samples were taken in each case immediately after the solutions were made up to the correct volume and before any appreciable enzymatic action could take place.

The scab organism was tested for its activity on the following substrates: Amygdalin, salicin, sucrose, hydrogen peroxide, olive oil, casein, gelatin, urea, starch, and cellulose.

EXPERIMENTAL DATA

GLUCOSIDASE

The enzyme material was tested for its glucoside-hydrolyzing power by using as substrates amygdalin and salicin. For each test 1 gm. of the glucoside was dissolved in about 60 c. c. of distilled water in a 100 c. c. volumetric flask. To this was added an enzyme mixture representing 0.1 gm. of dry material. The solution was then brought up to volume in the flask, thus giving concentrations of substrate and enzyme material of 1 and 0.1 per cent, respectively. The digestion mixtures were tested at various times with Fehling's solution to determine the amount of dextrose present, which had been formed by the hydrolysis of both amygdalin and salicin. The results are given in Table 1.

TABLE 1.—*Hydrolysis of amygdalin and salicin by the enzyme material prepared from Gibberella saubinetii*

HYDROLYSIS OF AMYGDALIN			HYDROLYSIS OF SALICIN		
Time	Quantity hydrolyzed	*K*	Time	Quantity hydrolyzed	*K*
	Per cent			*Per cent*	
23 hours	10.79	0.0049654	16 hours	10.05	0.006621
30 hours	13.63	.0048854	24 hours	15.07	.006807
48 hours	18.47	.0042548	48 hours	22.86	.005410
55 hours	20.04	.0040671	65 hours	28.06	.005146

Both of these glucosides were quite readily hydrolyzed by the enzyme material. No attempts were made to determine the time necessary for the hydrolysis to stop or go to completion. The reaction constants

$$K = 2.303 \frac{1}{t} \operatorname{Log}_{10} \frac{a}{a-x}$$

are fairly uniform in each experiment, especially in that of the hydrolysis of amygdalin. As shown by the reaction constants, salicin was hydrolyzed more rapidly than amygdalin. Generally speaking, the constants decreased slightly toward the end of each experiment.

INVERTASE

Four grams of cane sugar was dissolved in about 60 c. c. of distilled water in a 100 c. c. volumetric flask. To this was added 0.1 gm. of enzyme material, as in the previous experiment. The solution was then brought up to a volume to give a concentration of 4 per cent sucrose and 0.1 per cent enzyme material. The amount of hydrolysis was determined at various times by making reduction tests with Fehling's solution. The results of the experiment are given in Table 2.

TABLE 2.—*Hydrolysis of sucrose by the enzyme material prepared from Gibberella saubinetii*

Time	Quantity hydrolyzed	K	Time	Quantity hydrolyzed	K
	Per cent			*Per cent*	
4 hours	12.68	0.033856	24 hours	57.70	0.035856
10 hours	28.10	.032952	30 hours	67.50	.037472
22 hours	53.95	.035303	34 hours	68.75	.034217

The table shows that the material contains a very active enzyme capable of hydrolyzing sucrose. The hydrolysis progressed rapidly and at a fairly definite rate, as shown by the velocity constants. Even after 34 hours and 68 per cent hydrolysis of the sucrose, the velocity of the reaction had not decreased to a point below the average rate of the reaction.

CATALASE

The reaction of the enzyme catalase was determined by measuring the volume of oxygen gas liberated from hydrogen peroxide (*10*). One-tenth gram of the enzyme material was added to 25 c. c. of 3.6 per cent hydrogen peroxide, and the gas evolved was collected and measured over water in a gas burette. Table 3 shows the activity of the enzyme material.

TABLE 3.—*Catalase activity of the enzyme material from Gibberella saubinetii as determined by the liberation of oxygen gas from hydrogen peroxide*

Time	Oxygen gas liberated	$\frac{x}{t}$	$K=\frac{x}{\sqrt{t}}$
	c. c.		
2 minutes	3.8	1.90	2.67
4 minutes	4.8	1.20	2.40
6 minutes	5.2	.87	2.12
8 minutes	5.4	.67	1.92

The velocity of the reaction decreases as the time increases. This reaction does not follow the monomolecular law but approaches Schütz's rule. Considering the fact that only 0.1 gm. of enzyme material was used, Table 3 shows that the enzyme catalase in the material is relatively active. After eight minutes, however, the reaction stopped, since no more oxygen was evolved.

LIPASE

The enzyme material was tested for its lipolytic activity by using an emulsion of olive oil at pH 4.7, which is suggested by Willstätter and Waldschmidt-Leitz (*5, p. 16*). Fifteen cubic centimeters of the emulsion were placed in each of four test tubes, and to each tube was added a quantity of a solution representing 0.1 gm. of dry enzyme material. At the time indicated each tube was titrated according to the method of Willstätter and Waldschmidt-Leitz (*11*) to determine the acidity produced by the hydrolysis of the oil. The percentage of oil hydrolyzed is shown in Table 4.

TABLE 4.—*Hydrolysis of olive oil by the enzyme material prepared from Gibberella saubinetii*

Test-tube No.	Time	Oil hydrolyzed	$K=\frac{x}{\sqrt{t}}$
	Hours	*Per cent*	
1	0	0	
2	24	1.48	0.30
3	48	2.36	.34
4	72	2.95	.35

There was some hydrolysis of the oil, even though the reaction was not very strong. It must be noted, however, that since the oil was partially hydrolyzed, the reaction cannot be disregarded. The reaction diminished as the time progressed, with considerable retardation on the third day. The reaction does not follow the monomolecular law, but when Schütz's rule is applied it is found that the transformation is proportional to the square root of the time.

PROTEASES

The proteolytic activity of the enzyme material was determined by using both casein and gelatin as substrates. The casein solution was prepared by suspending 4 gm. of casein in 160 c. c. distilled water, adding 16 c. c. 0.2N NaOH and then heating to about 40° C. Then it was heated to 95° C. to inactivate the enzymes and bacteria present in the solution. This solution is diluted to approximately 200 c. c. and adjusted to pH 9. Enzyme material corresponding to 0.2 gm. of dry material was added, and the solution was made up to 200 c. c. with distilled water. The same procedure was repeated for the tests at pH 6 and at pH 3. In the preparation of the gelatin solution 2 gm. were dissolved in about 50 c. c. water, diluted to approximately 100 c. c. and sterilized. To this, NaOH solution was added to bring the solution to pH 6, and enzyme material added corresponding to 0.1 gm. of dry material and the volume made up to 100 c. c. The hydrolysis of these proteins was measured by determining the increase of both the monoamino acids and peptones by precipitating with 5 per cent phosphotungstic acid and saturated zinc sulphate solutions, respectively.

The phosphotungstic acid does not precipitate the monoamino acids in solution, but it does precipitate all other proteins, including the diamino acids. The saturated zinc sulphate solution precipitates all the proteins except the amino acids, peptids, and peptones. By determining the total nitrogen left in the filtrates after precipitation,

the amount of nitrogen in each group and the amount of nitrogen not precipitated by phosphotungstic acid and saturated zinc sulphate solution may be calculated. The amount of nitrogen not precipitated by zinc sulphate minus the amount not precipitated by phosphotungstic acid gives the amount of nitrogen in the form of diamino acids, peptids, and peptones, which are frequently designated collectively as peptones.

TABLE 5.—*Hydrolysis of casein and gelatin by the enzyme material prepared from Gibberella saubinetii*

pH	Time	N not precipitated by phosphotungstic acid	N not precipitated by zinc sulphate	N as peptones
	HYDROLYSIS OF CASEIN			
	Hours	*Per cent*	*Per cent*	*Per cent*
9	0	2.63	8.74	6.11
	24	4.72	12.93	8.21
	48	6.82	15.03	8.21
	72	6.82	17.14	10.32
6	0	2.63	8.74	6.11
	24	6.13	12.26	5.13
	48	9.63	15.74	6.11
	72	12.25	19.25	7.00
3	(a)			
	HYDROLYSIS OF GELATIN			
6	0	2.62	4.50	1.88
	48	6.82	21.00	14.18

[a] No action.

Table 5 shows that there was considerable hydrolysis at both pH 9 and pH 6. There was more hydrolysis of the casein at pH 6 than at pH 9 and no hydrolysis at pH 3. This would indicate that the enzymes in this organism are of the tryptic and ereptic types and possibly a pepsinase, which the results at pH 6 suggest.

The gelatin was quite rapidly hydrolyzed at pH 6, and the enzyme material was more active in the formation of peptones from gelatin than from casein at pH 6.

UREASE, AMYLASE, AND CELLULASE

Tests were made for the action of urease, amylase, and cellulase by using urea, starch, and cellulose, respectively, as substrates, but no action was obtained from any of them. The fact that these enzymes were not found to be active in the dry enzyme material does not prove that they were not present in the living organism.

SUMMARY

In the study of the organism *Gibberella saubinetii* a medium was used that consisted of 4 per cent malt extract and 0.2 per cent nitrate of soda, on which the scab fungus grew in a manner to meet the requirements for the enzyme studies of the dehydrated material.

The hydrolysis of the glucosides amygdalin and salicin by the enzyme material showed a decided activity, indicating the presence of glucosidase.

The hydrolysis of sucrose by the enzyme material was much more rapid than that of the glucosides by glucosidase, as shown by the respective velocity constants, and the presence of invertase was indicated.

The enzyme material showed a decided catalase reaction.

The action on olive oil was weak but sufficient to prove the presence of lipase.

The proteins casein and gelatin were used as substrates to study the proteolytic properties of the extract. Casein was hydrolyzed readily at pH 9 and pH 6, but not at pH 3, indicating the presence of proteolytic enzymes, especially trypsin and erepsin. The results for gelatin are in general agreement with those for casein.

No positive results were obtained for urease, amylase, and cellulase.

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