Effect of Phenolic Acids on Alpha-Amylase in Vitro, and Early Growth of Sugarbeet[1]

J. M. Sebeson[2], Earl Mitchell[3], and F. W. Snyder[2]

Varner, et al. (4)[4] have reported an in vitro procedure for measuring α-amylase activity upon starch solutions. The reserve energy for the germinating sugarbeet embyro is the starchy endosperm or perisperm. Therefore, at least one enzyme would be required to hydrolyze the starch into a utilizable form. Juliano[5] has extracted from sugarbeet seeds (germinated four days) and identified by means of gel electrophoresis the enzyme α-amylase.

Since 1957, a number of potentially inhibitory phenolic acids have been isolated from sugarbeet fruits and identified. Five of those reported, caffeic (1,2), ferulic (1,2), gallic (3), p-hydroxybenzoic (1,2), and vanillic (2) acids were evaluated for their inhibitory effect on α-amylase activity in vitro. An attempt also was made to ascertain the degree of inhibition of these phenolic acids on germination of seeds excised from the fruits of sugarbeet.

Methods and Materials

The α-amylase solution from barley and the sodium acetate buffer, iodine reagent, and starch solution were prepared according to the methods of Varner, et al. (4).

Aqueous solutions of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}M caffeic, ferulic, gallic, p-hydroxybenzoic, and vanillic acids, and 10^{-2}M, gallic acid were used. The latter concentration was unattainable with the other acids because of their insolubility in water.

The incubation procedure was as follows: To 0.1 ml of α-amylase (containing 10 mg protein/ml), add 0.1 ml of phenolic acid, then add 0.8 ml of sodium acetate buffer (pH 4.82) and incubate at 25 C for 60 minutes. The phenolic acid was omitted from the control.

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[2]Associate Professor, Department of Chemistry, Lansing Community College, Lansing, and Plant Physiologist, Crops Research Division, ARS, U.S. Department of Agriculutre, East Lansing, Michigan.

[3]Graduate student, Department of Biochemistry, Michigan State University, East Lansing, Mich., now Assistant Professor, Department of Biochem., Oklahoma State University, Stillwater, Okla.

[4]Numbers in parentheses refer to literature cited.

[5]Dr. Bienvenido Juliano, Research Associate, MSU/AEC, Plant Research Laboratory, East Lansing, Michigan.

For the assay, 0.1 ml of the test aliquot was removed from the incubation medium and diluted to 1 ml with sodium acetate buffer. The reaction was started by adding 1 ml of starch solution and allowed to run at room temperature for exactly 4 min. The reaction was stopped by adding 1 ml of iodine reagent. After adding water to make a final volume of 8 ml, the contents of the test tube were thoroughly mixed. The optical density was determined in a colorimeter at 620 mμ.

The computations of $\Delta A_{620m\mu}$, units of activity, and the percent of activity were determined as follows:

$$\Delta A = A_b - A_s$$

where A_b = absorbance of the starch-iodine blank
and A_s = absorbance of the sample

$$U = \frac{\Delta A \times V}{a \times t}$$

where U = units of activity
V = volume (in this case 1 ml)
a = aliquot (0.1 ml)
t = time in minutes

$$P = \frac{Ui \times 100}{U_e}$$

where P = percent of activity
Ui = units of activity with enzyme plus inhibitor
U_e = units of activity with enzyme

The activity of the pure enzyme was 2.85 or 100%. The percent of inhibition of the phenolic acids was assumed to be the percent of activity subtracted from 100%.

The influence of calcium ions and dithiothreitol on the activity of α-amylase in the presence of 5×10^{-4} M gallic acid was determined. Crystalline α-amylase was incubated either with or without gallic acid. The assay was for 1 min. The assay medium consisted of 0.1 ml enzyme solution (25 mg of protein) and 0.9 ml of one of the following buffer solutions:

1. Control—0.001M acetate buffer with 0.01M $CaCl_2$ pH 4.8.
2. High Calcium—0.001M acetate buffer pH 4.8 with 1.0M $CaCl_2$.
3. Dithiothreitol—0.001M acetate buffer pH 4.8 with 0.01M $CaCl_2$ and 0.2M dithiothreitol.

Sugarbeet seeds, excised from the fruits, were placed on a filter paper in a Petri dish and the solution of phenolic acid was added. Concentrations used were 10^{-3} and 10^{-4} M, except caffeic acid did not dissolve completely at the 10^{-3} concentration at room temperature. Thus, the caffeic acid at the higher concentration was a saturated solution and at the lower concentration it was

diluted ten-fold. The limited supply of excised seeds permitted only three replications of 10 seeds each for the higher concentration and two replications of eight seeds each for the 10^{-4} M treatments. Root lengths of the seedlings were measured after 5 days.

Results

The data (Table 1) indicate that α-amylase activity in vitro is inhibited most strongly by gallic acid. In the range of 10^{-3} to 10^{-5} M concentrations, caffeic and p-hydroxybenzoic acids were intermediate in inhibition, while ferulic and vanillic acids were least inhibitory.

Table 1.—Effect of five phenolic acids on α-amylase activity in vitro.

Conc.	acid	$A_{620\ \mu}$	$\Delta A_{620\ \mu}$	Units	Percent act.	Percent inhibit.
10^{-3}M	Gallic	1.35	0.13	0.32	11.2	88.8
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	Ferulic	0.85	0.63	1.57	55.0	45.0
	Vanillic	0.89	0.59	1.47	51.6	48.4
	Caffeic	0.98	0.50	1.25	43.8	56.2
	p-Hydroxy benzoic	1.19	0.29	0.67	23.8	76.2
10^{-4}M	Gallic	1.35	0.13	0.32	11.2	88.8
	Ferulic	0.76	0.72	1.80	63.1	36.9
	Vanillic	0.78	0.70	1.75	61.4	38.6
	Caffeic	0.93	0.55	1.37	48.0	52.0
	p-Hydroxy benzoic	0.93	0.55	1.37	48.0	52.0
10^{-5}M	Gallic	0.85	0.63	1.57	55.0	45.0
	Ferulic	0.72	0.76	1.90	61.7	38.3
	Vanillic	0.77	0.71	1.77	62.0	38.0
	Caffeic	0.80	0.68	1.70	59.6	40.4
	p-Hydroxy benzoic	0.82	0.66	1.65	57.8	42.2
10^{-6}M	Gallic	0.71	0.77	1.92	67.3	32.7
	Ferulic	0.65	0.83	2.07	72.5	27.5
	Vanillic	0.52	0.96	2.40	84.2	15.8
	Caffeic	0.65	0.83	2.07	72.5	27.5
	p-Hydroxy benzoic	0.71	0.77	1.92	67.3	32.7
Control		0.34	1.14	2.85	100.	
Blank		1.48				

The marked inhibition of α-amylase activity by gallic acid was not reversed by calcium ions (required for enzyme stability) or by dithiothreitol (protects the SH groups). The % inhibition versus incubation time curve is given in Figure 1.

The effect of the five phenolic acids on germination and growth of the intact sugarbeet seedlings was much less striking. Root lengths were variable but roots of some of the treated seedlings were long and appeared to be uninhibited by the phenolic acids.

We have estimated the concentration of gallic acid in sugarbeet fruits to be in the range of 10^{-4} M. This concentration of gallic acids inhibited α-amylase activity in vitro by 89%. Concentrations of the other acids in the fruits have not been estimated.

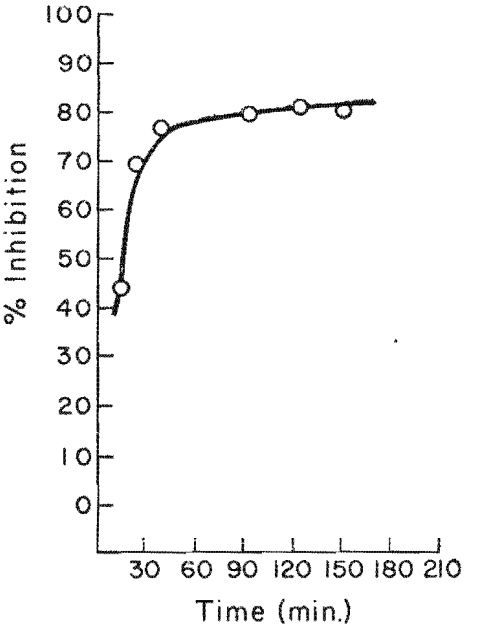

Figure 1.—Inhibition of alpha-amylase by gallic acid when incubated with 5×10^{-4}M gallic acid plus 0.01 M Ca^{++} and 0.01 M dithiothreitol.

Discussion

The presence of substances in sugarbeet fruits which delay or inhibit germination of the seed, the identification of a number of these potentially inhibitory substances, and the development of the test for α-amylase activity in vitro provide a system for attempting to evaluate the importance of some of these substances in affecting germination and early growth.

The kinetics of inhibition by the phenolic acids in the in vitro system have not been investigated. However, it is possible that these phenolic compounds bind irreversibly with the enzyme, perhaps at the site of activity. Adding calcium and dithiothreitol did not alter the inhibition.

The inherent variability in the length-growth of sugarbeet roots complicates any statistical analysis of data when the treatments do not cause large differences in growth. On the basis of average length of roots, the phenolic acids at concentrations of 10^{-3} M retarded root growth more than at 10^{-4} M. Although the variable root growth precludes any statistical significance, the trend may be indicative of increasing toxicity with increasing concentration.

Rate of germination and subsequent root growth of sugarbeet were only slightly retarded, but α-amylase activity in vitro was markedly inhibited (89%) by 10^{-3} M gallic acid. This disparity is very interesting, particularly since repeated efforts have been made to demonstrate a significant inhibitory effect of gallic acid on germination and subsequent growth. How can the observations be reconciled? If the 89% inhibition of α-amylase activity occurs in the seed, it seems doubtful that sufficient hydrolyzed material would be available to essentially permit as rapid growth as in a water-control. If this did occur, it would suggest a very large excess of α-amylase activity for the normal situation. Perhaps the degree of inhibition imposed by gallic acid (as well as the other phenolic acids) on α-amylase in vitro does not occur in the intact seed. Possibly the cells contain a mechanism which would detoxify these phenolic acids and thereby drastically reduce the concentration of the inhibitory substance.

Should some such mechanism as the above account for the above noted disparity, the dangers inherent in extrapolating the effect of substances on isolated enzyme systems to intact organisms become very apparent. The effect on the intact organism, therefore, must be determined directly through experimentation on that organism.

Summary

The effect of caffeic, ferulic, gallic, p-hydroxybenzoic, and vanillic acids on α-amylase activity in vitro at molar concentrations of 10^{-3} to 10^{-6} was determined. Gallic acid was most inhibitory. Caffeic and p-hydroxybenzoic were intermediate, while ferulic and vanillic acids were least inhibitory in the range of 10^{-3} to 10^{-5} M. At 10^{-4} M, they ranged between 37 and 89%; at 10^{-5} M between 38 and 45%, and at 10^{-6} M between 16 and 33%. and at 10^{-6} M between 16 and 33%.

Sugarbeet seeds, excised from the fruits, were placed in 10^{-3} and 10^{-4} M solutions of the five acids. Root growth after 5 days was measured. Root lengths tended to be shorter at 10^{-3} than at 10^{-4} M, but not statistically significant. The marked inhibition of the acids on α-amylase activity in vitro does not occur in the intact seed during germination and subsequent growth.

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