Gallic Acid in Sugarbeet Fruits[1]

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Approximately ten organic compounds isolated from the fruits of sugarbeets, which may be potentially inhibitory to rate of germination, have been reported in various publications (10)[3]. Massart (6) identified by paper chromatography, vanillic, ferulic, p-hydroxybenzoic, and cinnamic acids in aqueous extracts of sugarbeet fruits and showed that they delay or inhibit germination. Köves and Varga (4), also using paper chromatography, identified caffeic, ferulic, p-hydroxybenzoic, p-coumaric and salicylic acids in an ether extract obtained from the water extract of sugarbeet fruits. They were unable to demonstrate the presence of vanillic acid, but they did observe an unknown inhibitor. Recently, Mitchell (7) identified cis-4-cyclohexene-1,2-dicarboximide in sugarbeet fruits and determined that it is inhibitory to lettuce and sugarbeet seeds. Makino and Miyamoto (5) and Miyamoto (8) identified oxalate in sugarbeet fruits and established that oxalate was inhibitory to germination. A statistically significant relationship ($r = -0.6^{**}$) was found between the quantity of water soluble oxalate in the fruit and speed of germination of sugarbeet (10). Evidence for an inhibitory substance(s), other than oxalate, also was obtained in this study (10). Furthermore, the phenolic carboxylic acids appear to play a role in the resistance of the sugarbeet to *Cercospora* leaf spot disease (2).

This paper reports the identification of gallic acid (3,4,5-trihydroxybenzoic acid) in various extracts of sugarbeet fruits by means of thin-layer chromatography (TLC), color reactions, and ultra-violet (UV) light interaction. The amount of gallic acid in the fruit extract was estimated and the influence of gallic acid on germination was determined.

Methods and Materials

Isolation

Extracts of air-dried fruits of a commercial monogerm sugarbeet variety were prepared by eight different procedures. The details of three of the procedures follow.

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[3] Numbers in parentheses refer to literature cited.

Extract No. 1

Ten grams of fruits were soaked in 50 ml of water for 18 hours in a refrigerator. The pH of this extract was adjusted to 5 to 6. To 25 ml of extract, 15 ml of 15% $CaCl_2$ was added and the mixture was heated at 80 C for 1½ hours to promote precipitation. The precipitate was filtered and dissolved in 25 ml of 5% HCl. This solution was extracted three times with 15 ml of ether. The ether extracts were evaporated to a volume of 5 ml to concentrate the solutes.

Extract No. 2

Ten grams of fruits were digested with 30 ml of 5% HCl for 4 hours. After filtration, the solution was made alkaline with NaOH and evaporated to dryness under reduced pressure with the temperature held under 50 C. The residue was dissolved in 10% HCl and extracted twice with 15 ml of ether, and then the ether volume was reduced to 5 ml.

Extract No. 3

Ten grams of fruits were placed in 50 ml of water and were heated at 80 C for 12 hours. The solution was filtered, made alkaline with 0.1N NaOH, and evaporated to dryness under partial vacuum at a temperature below 50 C. The residue was dissolved in 10% HCl and extracted three times with 20 ml of ether. The ether extract was neutralized with 0.1N NaOH and evaporated to the aqueous phase (1 ml). The aqueous portion was acidified with H_2SO_4 and passed through a column packed with chromatographic grade Silica (50-200 mesh, G. F. Smith)[4]. The column was eluted with four fractions (a, b, c, d) of 5, 10, 20 and 30% 1-butanol in chloroform. Gallic acid was found in the third fraction (c). This method was adapted from one used by Goncharova (1).

The other procedures included various combinations of those given above, and in addition, initial soaking in 95% ethanol and final extraction in acetone.

The TLC stationary phase (0.25 mm in thickness) was made of Silica Gel G (Merck, Germany), using 30 g of silica gel in 60 ml of water, according to directions in the Operating Manual 103-B of Desaga/Brinkmann[4]. Because the presence of water in the thin-layer materially affects the R_f values, the plates were dried in an oven at 105 C for 2 hours. The dried plates were stored in a desiccator with potassium hydroxide pellets as the dehydrating agent. Before use, the plates were again heated in an oven at 105 C for 1 hour.

[4] The use of specific brand names and procedures does not indicate endorsement of product to the exclusion of others, but indicates procedural methods used.

For the majority of the determinations dried plates were used. In others, the internal water phase of the plates was increased by steaming the layers after spotting and before irrigation, according to the methods employed by Van Sumere, *et al.* (12). The plates were held over a beaker of boiling water until they were uniformly wet. (Care must be used here because oversteaming can destroy the thin-layers). After steaming, the plates were dried at room temperature for about 5 minutes before irrigation was started. (A double front will result if the steamed layer is used immediately.) The R_f values of the phenolic acids are dependent upon the water content of the thin-layers; hence, to get reproducible values, the steaming and the drying of the plates must be carried out under the same conditions each time.

Identification

The four most definitive (i.e. - they showed the greatest amount of separation among the spots) solvent systems used in the TLC procedure were as follows: 1) ethanol, ammonia, water (100:12:16 v/v); 2) benzene, methanol, glacial acetic acid (90:16:8); 3) benzene, dioxane, glacial acetic acid (90:25:4); and 4) methanol, 5N ammonium hydroxide (80:20).

The irrigation of the thin-layer plates was done in a standard Desaga glass chamber fitted with a ground glass cover. The time for development (10-15 min.) is short and is one of the advantages of TLC.

The developed spots were sprayed with bromcresol green (40 mg per 100 ml H_2O adjusted to a blue color with 0.1N NaOH), 1% ferric chloride, or diazotized p-nitroaniline. This latter spray was prepared by mixing 5 ml p-nitroaniline (0.5% in 2N HCl) and 0.5 ml $NaNO_2$ (5%, v/v), and then adding 15 ml sodium acetate (20%, v/v). Acidic compounds react with bromcresol green to produce yellow to green colors depending upon the degree of acidity. Ferric chloride produces shades of gray to brown. Best results were obtained with bromcresol green and ferric chloride sprays.

Poly-hydroxy aromatic compounds will fluoresce or absorb under UV light in the wavelength range 230-290 mμ, depending upon the substituent group or groups attached to the benzene ring, in addition to the OH groups. Some spots develop color after being sprayed with a 2N sodium hydroxide solution. Gallic acid is a pink-violet under UV light; the color is intensified after spraying with sodium hydroxide.

Effect on germination

Three experiments were conducted to ascertain any inhibitory effect of gallic acid on germination and early seedling growth

Table 1.—Chromatographic data for extracts of sugarbeet fruits developed in four solvent systems using TLC.

	Solvent system*							
	1		2		3		4	
Spot	R_f	Color**	R_f	Color**	R_f	Color**	R_f	Color***
1	0.11	Green	0.094a	Yellow	0.90a	Green	0.11	Decolorized
2	0.17a	Yellow	0.195	Yellow	0.20b	Yellow	0.17	Decolorized
3	0.27	Yellow	0.24b	Yellow	0.38a	Yellow	0.27	Grey
4	0.35a	Green	0.35a	Green	0.636a	Yellow	0.35a	Brown
5	0.52	Yellow	0.39a	Green	0.73a	Yellow	0.52a	Brown
6	0.59b	Yellow	0.56	Green	---	---	0.68b	Brown
7	0.73a	Green	0.83a	Green	---	---	0.73a	Light Brown

* Composition given under Methods and Materials.
** Bromocresol green spray.
*** 1% ferric chloride spray.
a Spot fluoresced under UV light.
b Spot pink-violet under UV light and fulfills criteria for gallic acid.

of sugarbeets. Excised true seeds were placed on filter paper in Petri dishes to which was added 2 ml of either gallic acid solution or of distilled water for the control. Concentrations of gallic acid used were 10^{-4} and 10^{-3}M. Higher concentrations were not used since they would be unrealistically high in terms of the possible concentration in the fruit. Evaporation of liquid was avoided during the 4-day period of growth. Length of root was recorded for each seedling.

Results

Isolation and identification

A total of 180 chromatograms were made in this study. Usually six spots were placed on one plate. The volumes of extract applied to each spot varied from 10 to 200 μl.

After a number of exploratory chromatograms were made, we noted that one spot appeared with regularity on all chromatograms. The literature R_f values, color reactions, and the response to UV light indicated that the spot was gallic acid. Table 1 summarizes the results obtained. After preliminary indications that gallic acid separated on the chromatograms, pure gallic acid was spotted along with the extract spots. The R_f values of the pure gallic acid corresponded closely with the extract spots. Color and UV light reactions gave further positive proof of identification. The spot in each solvent system which corresponds to the position for gallic acid is indicated in Table 2, (11). Gallic acid absorbs UV light resulting in a pink-violet color and the test spots conformed closely. Also, due to the electron releasing substituents (the OH groups on the benzene ring) gallic acid should be a strong acid. The test spots reacted with bromcresol green to produce a yellow color, thus indicating strong acidity.

Since in the literature, ferulic, caffeic, oxalic, vanillic, and p-hydroxybenzoic acids have been reported as being present in

Table 2.—R_f values reported in the literature (11) for a number of acids which have been identified in extracts of sugarbeet fruits.

	Solvent system	R_f Value					
		Gallic acid	Ferulic acid	Caffeic acid	Oxalic acid	Vanillic acid	p-Hydroxy-benzoic acid
1	Ethanol, ammonia, water	0.59	0.52	0.36	0.098	0.55	0.44
2	Benzene, methanol, acetic acid	0.24	0.517	0.38	0.05	0.513	0.52
3	Benzene, dioxane, acetic acid	0.20	0.33	0.17	0.11	0.42	0.48
4	Methanol, ammonia	0.68	0.62	0.56	0.15	0.74	0.50

sugarbeet fruits, solutions of these acids and the extracts were spotted. In a number of the chromatograms, these acids appeared to be present in the extracts.

By using the same volumes and comparing the size of the spots with the sizes of the spots of pure gallic acid of known concentrations, it was possible to estimate the concentration of gallic acid present in the fruit. The concentration of free gallic acid in the extracts of the two seedlots analyzed was estimated to be 10^{-4}M.

A longer period of hydrolysis produced a higher concentration of gallic acid. Extract No. 2 had a high concentration, while samples soaked in 95% ethanol for 12 hours, which isolated only free gallic acid, produced the least. As would be expected, Extract No. 3 produced chromatograms with the best separations.

Up to six other acidic compounds of the phenolic type separated out on the chromatograms. As was pointed out above, some of these corresponded to ferulic, caffeic, vanillic, and p-hydroxybenzoic acids; some were not identified. This is an area for additional fruitful investigation.

Inhibitory action

Root length of 316 sugarbeet seedlings on the two concentrations of gallic acid and the water control did not differ significantly. A trend toward less root growth on 10^{-3}M gallic acid was noted, however. The root length of sugarbeet seedlings 4 days after the start of germination is usually inconsistent under any growth conditions. This variability actually prevents the attainment of statistically significant data with any manageable-sized experiment. In an attempt to reduce the variability, we used true seeds collected from a single plant which had loose seedcaps. The variability still was large and the differences were not significant.

Wheat grown, as a bioassay test (9), at the same concentrations of gallic acid as the sugarbeets also did not differ significantly from wheat grown in the water control.

The source of energy for the germinating sugarbeet embryo is starch. Thus, at least one enzyme is required to hydrolyze the starch into a substance which can be utilized by the embryo. To identify the hydrolyzing enzyme(s) in sugarbeet, Juliano[5] used endosperm of seeds 4 days after start of germination. Using the gel electrophoresis technique, he established that the hydrolytic enzyme is alpha-amylase.

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

Gallic acid at 10^{-1}M concentration markedly inhibited (nearly 90%) and at 10^{-6}M inhibited by one-third alpha-amylase activity in vitro[a]. Thus, the highly inhibitory effect of gallic acid on alpha-amylase activity in vitro is in sharp contrast with the statistically non-significant effect on germination and seedling growth in vivo.

Discussion

The literature abounds with evidence that gallic acid is present in numerous plants. Gallic acid readily forms 3-glucogallic, 4-glucogallic, and 5-glucogallic acids. Some gallic acid is found free in plants, but most of it is present as a glucoside.

Phenolic acids have been shown to inhibit germination significantly. The concentrations of the acids and phenols listed below cause a 50% inhibition of the germination of lettuce seeds (3).

Catechol	10^{-2}M	Ferulic acid	5×10^{-3}M
Resorcinol	5×10^{-3}M	Caffeic acid	$>10^{-2}$M
Salicylic acid	1.5×10^{-3}M	Coumaric acid	5×10^{-3}M
Gallic acid	5×10^{-3}M	Pyrogallol	10^{-2}M

Although in the above data (3), gallic acid of 5×10^{-3}M concentration inhibited 50% of the lettuce seeds from germinating, we were unable to demonstrate a statistically significant reduction in germination or seedling growth of sugarbeet in 4 days by placing seeds in 10^{-3}M gallic acid.

Gallic acid strongly inhibited the enzyme, alpha-amylase in vitro, but only slightly inhibited germination and seedling growth of sugarbeet. Although the enzyme is required to hydrolyze the reserve starch to provide energy for germination and growth, these data indicate the danger of extrapolating inhibitory effects on enzyme systems in vitro to the possible effect on germination of the seed and subsequent seedling growth.

Summary

Air-dried fruits of sugarbeet were extracted with water, ethanol, or 5% HCl. The solution was then extracted with ether. These extracts contained gallic acid, based on the R_f values obtained with four solvent systems in thin-layer chromatography, a pink-violet color and absorption under UV light, and color reactions for a strong acid.

The concentration of gallic acid in the samples analyzed was estimated to be approximately 10^{-1}M. A concentration of 10^{-3}M slightly inhibited germination and early root growth of sugarbeet. However, in vitro as little as 10^{-6}M gallic acid significantly inhibited alpha-amylase - the enzyme which hydrolyzes starch, source of reserve energy, for the germinating embryo of sugarbeet.

[a] Data of J. M. Sebeson, Earl Mitchell, and F. W. Snyder. See page 556

Literature Cited

(1) GONCHAROVA, I. A., A. N. KHOMENKO, and A. D. SEMENOV, (Hydrochem Inst., Novocherkassk). 1966. Determination of nonvolatile carboxylic acids in natural waters. Gidrokhim. Mater. 41: 116-20.

(2) HARRISON, M., M. G. PAYNE, and J. O. GASKILL. 1961. Some chemical aspects of resistance to Cercospora leaf spot in sugar beets. J. Am. Soc. Sugar Beet Technol. 11 (6) : 457-467.

(3) MAYER, A. M., and A. POLJAKOFF-MAYBER. 1963. The germination of seeds. In international series of monographs on pure and applied biology, Plant Physiology Division. Pergamon Press, New York, 236 pp.

(4) KOVES, E., and M. VARGA. 1959. Comparative examination of water- and ether-soluble inhibiting substances in dry fruits. Phyton 12: 93-99.

(5) MAKINO, I., and T. MIYAMOTO. 1954. On the growth inhibiting substance in germinating spinach seeds. Jap. J. Breeding. 4: 158-160.

(6) MASSART, L. 1957. Inhibiteurs de la germination dans les glomerules de la betterave a sucre et dans d'autres fruits secs et graines. Biokhimiia 22: 417-420.

(7) MITCHELL, E. D., JR., and N. E. TOLBERT. 1968. Isolation from sugar beet fruit and characterization of cis-4-cyclohexene-1,2-dicarboximide as a germination inhibitor. Biochem. 7 (3) : 1019-1025.

(8) MIYAMOTO, T. 1957. The germination inhibitor in sugar beet seedballs. Quart. Bull. Mich. Agr. Expt. Sta. 39: 518-523.

(9) SNYDER, F. W. 1963. Some physico-chemical factors of the fruit influencing speed of germination of sugar beet seed. J. Am. Soc. Sugar Beet Technol. 12 (5) : 371-377.

(10) SNYDER, F. W., J. M. SEBESON, and J. L. FAIRLEY. 1965. Relation of water soluble substances in fruits of sugar beet to speed of germination of sugar beet seeds. J. Am. Soc. Sugar Beet Technol. 13 (5) : 379-388.

(11) STAHL, EGON, and P. J. SCHORN. 1964. Thin-Layer Chromatography—A Laboratory Handbook. Academic Press, New York.

(12) VAN SUMERE, C. F., G. WOLE, H. TEUCHY, and J. KINT. 1965. A new thin-layer method for phenolic substances and coumarins. J. Chromatog 20: 48-60.