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Probable Identity of the Pectinase Inhibitor in Grape Leaves

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SUMMARY

Studies were continued on the chemical nature of the water-soluble substance in Muscadine grape leaves that was previously reported as inhibiting pectinase and cellulase. The inhibitor is a tannin or tannin-like material, removable from leaf extracts by hide powder, caffeine, gelatin, and nicotine sulfate. Muscadine leaves had the highest inhibiting activity of the plants tested.

INTRODUCTION

The importance in cucumber brining of the softening problem caused by enzymatic degradation of cell wall pectin was discussed by Etchells *et al.* (1958a, b). That paper also reported studies showing that the pectinolytic enzyme activity is chiefly the result of the growth of higher fungi (molds) in the cucumber flowers before they reach the brining station. Many of these flowers remain attached to the cucumbers when brined. Bell and Etchells (1958) and Bell *et al.* (1960) reported in Muscadine grapes (Scuppernong variety) a factor that inhibited pectinase and cellulase activity.

The grape leaf, inhibitor was found by Bell and Etchells (1958) to be water-soluble, stable to heat, non-dialyzable through a cellophane membrane, and not completely precipitable by acetone or concentrated ammonium sulfate. They also found that reduction in pectinase activity was directly related to the inhibitor concentrations used and that the reaction between pectinase, substrate, and inhibitor was that of competitive inhibition.

Pectic enzymes are destroyed by the usual enzyme and protein denaturants such as heat, heavy-metal salts, strong acids, and strong alkalis. Kertesz (1951) reviewed the pectinase literature and found that compounds such as tannins, glycine, and formaldehyde were reported as being inhibitory. Later work, however, did not confirm the earlier findings on the latter two compounds. Rahman and Joslyn (1953) studied a number of the usual inhibitors such as monoiodoacetic acid, mercuric chloride, sodium azide, sodium fluoride, ammonium arsenate, and sulfur dioxide against purified polygalacturonase (pectinase) and concluded that the enzyme was generally resistant or only slightly affected by these compounds.

Naturally occurring inhibitors have been previously reported for the hydrolytic enzymes. Inhibitors for amylases were found in wheat by Militzer *et al.* (1946), and in Leoti sorghum by Kneen and Sandstedt (1946) and Miller and Kneen (1947). Weurman (1953) was the first to report a thermolabile inhibitor of pectinase, which he precipitated from pear sap with acetone.

Many investigators have demonstrated the inhibition of enzyme activity by tannins. Ehrenberg (1954) reported that the phosphatase activity of leaves of Kalanchoe was

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inhibited by the natural tannin content of the plant. The inhibition was eliminated by adding a slightly chromated hide powder to the extract. Caneghem and Spier (1955) showed that tannin, phosphotungstic acid, sodium lauryl sulfonate, thiazole yellow, and polyphloretin phosphate inhibited the action of hyaluronidase. Ishimatsu and Kibesaki (1955) showed that agarase activity was inhibited by tannic acid. Friedrich (1955) reported that the natural tannin present prevented the plant enzymes from splitting arbutin during the extraction process, and hypothesized that such protective action probably accounted for the greater medicinal value of the extract over that of crystalline arbutin. Jackson and Wood (1959) demonstrated in rose hips an inhibitor that was effective against the oxidation of ascorbic acid, and postulated that tannin may be the active principle.

Other work has shown that tannins are effective inhibitors for pectinase. Grossmann (1958) reported that pectinolytic enzymes from *Fusarium oxysporum* f. *lycopersici* were inhibited by added tannins, that chebulinic acids were less effective, and that D-catechins were ineffective. Hathaway and Seakins (1958), reporting on the inhibition of pectinase by several tannins, showed that myrabolan tannin inhibited the rate of hydrolysis but that the hydrolysis went to completion if sufficient time was allowed. This suggests a competitive type of inhibition. Gambir tannin was shown to have no effect. Cole (1956) demonstrated the inhibition of pectinase by tannin and oxidized apple juice.

Cadman (1959) showed that extraction of minced raspberry leaves with 2.5% nicotine gave a tannin-free extract that was very poor as an inhibitor in the prevention of virus infection. Barnes (1956) showed that tannin can be precipitated from water solution by N-bases such as caffeine and antipyrine.

Screening studies by the authors, on pectinase and cellulase inhibition by 61 species in 37 families of plants, have been completed and prepared for publication. The present work was initiated to obtain further information on the chemical nature of the pectinase inhibitor substance(s) in grape leaves as compared to a selected group of the screened plant samples, including those with and without inhibitory activity.

MATERIALS AND METHODS

Plant materials. Samples of grape leaves and of other plant leaves were collected at North Carolina State College and vicinity in the summer of 1960. The samples were washed with water, surface dried, and placed in polyethylene bags. All samples were stored at −15°C or below.

Extractions. Weighed samples (40.0 g) of the frozen leaves were cut with scissors and placed with 300 ml of distilled water in a Waring blender (mention of company and trade names does not imply endorsement over others not named). Toluene (1 ml) was added as a preservative, and the blender was run 30 sec at low speed and then increased to high speed for 2.5 min. The slurry was filtered through four layers of cheesecloth on a Buchner funnel and washed with 150–175 ml water. The filtrate was made to 500 ml. For viscosity measurements (inhibitory power), aliquots of this solution were centrifuged 15 min at 3000 rpm. For tannin analyses, the solutions were filtered according to the filtration procedure employed in the official hide-powder method of the American Leather Chemists Assoc. (1957).

Enzyme solution. A new enzyme solution was prepared each day. A weighed sample (0.100 g) of Pectinol 10-M (Rohm and Haas Company, Philadelphia, Pa.) was made to 100 ml with water. An aliquot of this solution was diluted to a concentration of 0.0002 g/100 ml.

Enzyme substrate. Six g of sodium polypectate (Sunkist Growers, Ontario, California) was dissolved in 500 ml of 0.02*M* NaOH-citric acid buffer at pH 5.0 and 55°C by mixing in a Waring blender. The resulting solution was filtered through several layers of cheesecloth and preserved with 1 ml of toluene. This is the solution used by Bell *et al.* (1955).

Measuring enzyme and inhibitor activity. The viscometric method of Bell *et al.* (1955) was modified by employing a more concentrated enzyme solution and by taking measurements after a reaction time of 1 hr and 2 hr instead of 20 hr. Using 100 units of pectinase activity as equivalent to a 50% viscosity loss in 2 hr of reaction time, a table was set up relating loss in viscosity to units of pectinase activity. This table was calculated from a curve relating the log of pectinase activity units to the percent loss in viscosity. Since a 10-fold enzyme concentration and only a 2-hr, instead of a 20-hr, reaction time were employed, the viscosity-activity relationships reported in the original method were the same as those calculated for this work.

Standard enzyme activity (control) was measured by mixing enzyme solution with water (2:1 v/v) and using 1 ml of this mixture added to 5 ml of substrate at 30°C in an Ostwald-Fenske viscosity pipette (uncalibrated, No. 300). Inhibitor activity was measured by substituting one volume of the inhibitor solution for the water. The enzyme and inhibitor were mixed at least 15 min before the viscosity measurement was started. Correction was made for the flow time for pure water for each pipette.

Tannin analyses. Tannin analyses were made by the official hide-powder method of the American Leather Chemists Assoc. (1957). The percent of tannin and the tannin purity values were calculated by the following formula:

$$\% \text{ tannin} = \frac{(A - B)\ 100}{C}$$

$$\text{Tannin purity} = \frac{(A - B)\ 100}{B}$$

where A = g solids per ml of extract

B = g solids per ml of extract after hide-powder treatment

C = wt sample per ml extract.

RESULTS AND DISCUSSION

Influence of concentration of grape leaf on inhibitory power of extract. Forty grams of Creek grape leaves (Muscadine group) were extracted and made to 500 ml. Serial dilutions from 80 mg/ml to 0.6 mg/ml were made, and the resulting solutions were tested for inhibitory power. The data are presented in Fig. 1. All concentrations down to and including 4.8 mg/ml gave 95% or more reduction in pectinase activity. On the basis of the tannin content (hide powder) of this sample (16.5% on a moisture-free basis) the tannin concentrations ranged from 5.2 mg/ml to 0.04 mg/ml. The concentration corresponding to 4.8 mg leaf/ml was 0.31 mg tannin/ml. As pointed out by Bell and Etchells (1958), leaves from species of grapes other than Muscadine varieties have less inhibitory activity and the inhibition starts to drop at a much higher concentration of leaf material per milliliter.

Relationship of type and quantity of tannin to inhibitory power of plant extracts. The presence of pectinase inhibitors in plants other than the grape has been demonstrated (Bell *et al.*, 1961). Several of these plants were selected to span the range of inhibitor activity from 95% to 0%, for use in a study of the relationship of tannin (as determined by hide powder) to inhibitory activity. The results are reported in Table 1. In all cases the hide-powder treatment used in the tannin analyses removed essentially all of the active principle, and removal was completed by further additions. However, the inhibitory power of the untreated extract was not perfectly correlated with its tannin content, because of three factors.

The first factor is that an increase in tannin content beyond a certain point produces little or no increase in inhibitory power (Fig. 1). An extract of Muscadine grape leaves, if diluted from 80 mg leaf/ml to 4.8 mg leaf/ml, contained 0.3 mg tannin/ml and still produced about 94% inhibition. Undiluted iris leaf extract (0.3 mg tannin/ml) and sericea extract (0.6 mg tannin/ml), each prepared from 80 mg leaf/ml, produced 88% and 80% reduction in pectinase activity, respectively. This would indicate that these three plant sources, on a tannin basis, appear to be about equaly effective. However, on a fresh leaf basis (80 mg leaf/ml) the Muscadine grape leaves appear to have 8–17 times the inhibitory capacity, because of the higher tannin content of the grape leaves.

The second factor is the varying inhibitory power of different types of tannin. This difference in activity due to tannin source is exemplified by tests on commercial tannins. Myrabolan tannin had a relatively high ac-

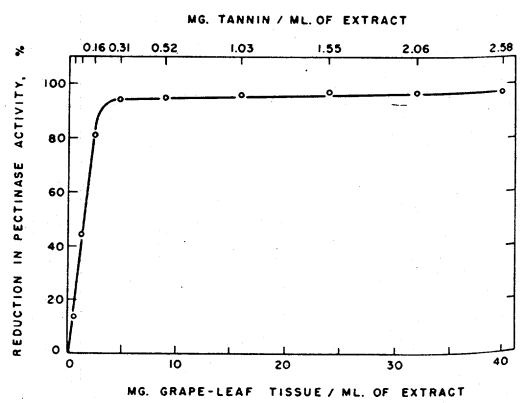

Fig. 1. Relationship of grape leaf concentration and tannin concentration to reduction in pectinase activity.

Table 1. Inhibitory activity of plants as related to tannin content.

Plant [a]		Moisture (%)	Tannin (MFB) (%)	Tannin in extract (mg/ml)	Tannin purity [b]	Inhibition of pectinase: Water extract (%)	Inhibition of pectinase: Non-tannin extract [c] (%)
(Plants with inhibitory activity)							
Muscadine Grape	*Vitis rotundifolia* var. Creek	60.0	16.5	5.3	43.7	95	7
Iris	*Iris* sp.	82.8	2.0	0.3	5.4	88	14
Sericea	*Lespedeza cuneata*	45.3	1.4	0.6	11.1	80	12
Raspberry	*Rubus strigosus* var. Latham	61.1	5.2	1.6	16.2	70	0
Strawberry	*Fragaria chiloensis*	72.6	7.4	1.6	19.6	60	0
Dogwood	*Cornus florida*	58.5	9.7	3.2	26.0	64	0
Peach	*Prunus persica*	59.7	2.9	0.9	9.4	60	0
Rose	*Rosa odorata*	61.8	3.9	1.2	14.2	57	14
Blueberry	*Vaccinium ashei*	54.3	19.3	7.1	42.6	54	21
Sweet Potato	*Impomoea batatas*	81.7	7.2	1.1	33.2	36	0
(Plants with negative to doubtful inhibitory activity)							
Tomato	*Lycopersicon esculentum*	86.3	2.6	0.3	6.7	12	3
Pear	*Pyrus communis*	55.9	6.0	2.1	19.4	11	19
Cherry	*Prunus cerasus*	63.9	2.4	0.7	8.8	0	13
Holly	*Ilex cornuta* var. Burfordii	62.1	4.1	1.2	20.2	0	4
Apple	*Pyrus malus*	53.0	4.9	1.8	12.4	0	8
Magnolia	*Magnolia grandiflora*	51.5	3.7	1.4	14.1	2	16
Fig	*Ficus carica*	68.6	4.0	1.0	11.9	0	0

[a] All samples consisted of 80 g fresh leaf tissue extracted 3 min in Waring blender at high speed with 600 ml of water, filtered through cheesecloth, and made to one liter, including washings.

[b] $\text{Tannin purity} = \frac{\%\ \text{tannin}}{\%\ \text{total solids}} \times 100$

[c] This column indicates residual inhibitory power after treatment with hide powder for tannin analysis. Further hide-powder additions produced extracts having no residual inhibition of pectinase.

tivity, and Wattle tannin had little or no activity. This confirms the work of Hathaway and Seakins (1958). It would appear that Muscadine grape, iris, and sericea may contain the same type of tannin. Blueberry, which gave only 54% reduction in activity though its extracts contained 7.1 mg tannin/ml, may be a second type of tannin. Montmorency cherry, holly, apple, and fig, which gave no reduction in activity, though their extracts contained 0.7–1.8 mg tannin/ml, probably contain tannins different from any of those above. It is apparent that a tannin with a low inhibitory efficiency would produce less enzyme activity reduction than Fig. 1 would predict, since the curve is apparently based upon a tannin of high efficiency.

The third factor, tannin purity (% tannin × 100/total solids), complicates the above considerations. Tannin extracts of low purity are known to give high tannin values by the hide-powder method, because of adsorption of non-tannins by the hide powder. Since iris and sericea gave water extracts of much lower purity than the Muscadine grape leaves, the actual tannin content may be lower than indicated by the analytical method. This would indicate that the tannin from these two sources may be more effective than the actual data show. For this and other reasons, further work is in progress on the identity of the types of tannins of high inhibitory capacity.

Influence of extractant on the inhibitory power of the extracts. A sample of Seuppernong grape leaves (Muscadine) was extracted in the usual manner with water. A second sample was extracted with 1.3% caffeine solution, and a third was extracted with 2.5% nicotine sulfate solution. The

water extract gave 95% inhibition, and the two alkaloid extracts gave no inhibition. Addition of caffeine to the water extract followed by centrifugation gave a solution producing no inhibition. Caffeine and nicotine sulfate alone had no influence on the rate of viscosity change due to the enzyme. Addition of gelatin to the leaf sample while extracting with water gave an extract having no inhibitory power.

Influence of treatment of water extracts on inhibitory power. The dialysis experiments of Bell and Etchells (1958) were repeated in more detail. An extract of Scuppernong grape leaves was prepared and diluted to a concentration of 2 mg leaf/ml. This concentration produced 80% inhibition before dialysis. The extract was dialyzed with stirring against distilled water for 2 hr, with four changes of the water outside the cellophane membrane. The dialyzed solution gave 82% inhibition, and the dialysate, after concentration *in vacuo* at room temperature to a volume equal to the starting volume, gave 7.5% inhibition. An extract containing 40 mg leaf/ml gave 98% inhibition before and after dialysis, and the dialysate gave 9% inhibition. The inhibition of the dialysate is insignificant when considered with the total inhibition. Therefore, the active inhibitor must be of relatively high molecular weight.

Adsorption on Amberlite IRA-410 (OH^-) completely removed all inhibitory materials. Elution with ammonium hydroxide and deionization with Dowex 50 (H^+) produced a dark solution that yielded a strong test for polyphenols with ferric chloride and showed an inhibitory power about 25% of that of the original extract.

A borate complex was made and adsorbed on Duolite A-4 resin charged with 0.1*M* $Na_2B_4O_7 \cdot 10\,H_2O$. All inhibitory activity was removed, and the ammonium hydroxide and sodium hydroxide eluates from the column were completely inactive. This indicates that the borate inactivates the tannin.

Using the technique employed by Bell and Etchells (1958) on grape leaf extract, we have been able to show that pectinase inhibition by myrabolan tannin is of a competitive nature.

In a preliminary gel filtration resolution with Sephadex G-50 (Pharmacia, Uppsala, Sweden), a grape leaf extract indicated 5–6 compounds, represented by elution peaks, as shown by the activity of the fractions as inhibitors of pectinase. Ferric chloride tests on the individual fractions indicate a close correlation between the polyphenol content and the inhibitory activity. Since the peaks were very quickly eluted from the column, it must be assumed that the active components were of relatively high molecular weight. The fact that multiple peaks of activity were obtained indicates a range of molecular weights.

All of the experimental work on the relation of tannin to inhibitory activity—removal of activity by hide powder, gelatin, and alkaloids; dialysis; ion-exchange studies both on natural extracts and on borated extracts; resistance to heat; and the relation of polyphenols to inhibitory power after crude separation according to molecular weight by means of gel filtration—indicates that the active inhibitor of pectinase found in Scuppernong grape leaves is a tannin or a tannin-like compound. There is always the possibility that some non-tannin compound, having many of the physical and chemical characteristics of the tannins, may be responsible, but, considering the number of positive correlations, this chance seems remote. Work is continuing on characterization of the type of tannin.

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