PECTINASE INHIBITOR IN GRAPE LEAVES[1]

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Introduction

Pectin and cellulose are two plant substances which are extremely important in studies relating to texture and quality of fruits and vegetables (**3, 14**). In general, cellulosic substances make up the primary structural element in the cell walls of plants, and these cells are held together by pectic materials. It has been estimated (**16**) that cellulose embraces about one-third of all plant matter; pectic substances, although somewhat less concentrated, are also extremely widespread. The enzymatic hydrolysis of these polysaccharides is generally brought about by two fungal enzyme systems, polygalacturonase and cellulase.

This study deals with a water-soluble, thermostable substance found in grape leaves which inhibits the action of purified polygalacturonase (PG), and also with a crude enzyme mixture from cucumber flowers referred to here as pectinase. PG catalyzes the hydrolysis of glycosidic bonds between deesterified galacturonide residues in pectic substances, with the formation of reducing groups and a simultaneous loss of viscosity (**5**).

The second enzyme system mentioned, cellulase, is also inhibited by the water-soluble extract from grape leaves; however, a separate report will be made on the properties of the cellulolytic enzyme inhibitor.

Pectic enzymes are inhibited by the usual enzyme and protein denaturants such as heat, salts of heavy metals, strong acids, and strong alkalies. KERTESZ (**7**) in 1951 reviewed the literature on pectinase inhibitors and found only a few incidental references on the subject; materials such as tannin, glycine, and formaldehyde were reported as being inhibitory. However, later studies reviewed did not confirm the earlier findings on the above inhibitors for pectinase. A number of the usual enzyme inhibitors such as monoiodoacetic acid, mercuric chloride, sodium azide, sodium fluoride, ammonium arsenate, and sulfur dioxide were tested against purified PG by RAHMAN and JOSLYN (**13**); they concluded that the enzyme was, in general, resistant or only slightly affected by these compounds.

JANSEN *et al.* (**6**) observed that a high concentration of crude pectinesterase prepared from orange flavedo was inhibitory both to purified PG and to a commerical enzyme preparation of pectinase, Pectinol 100 D. They were of the opinion that the inert protein accompanying the pectinesterase was probably responsible for the inhibition.

A number of naturally occurring inhibitors have been reported (**8, 9, 11, 12, 15**) for some of the hydrolytic enzymes. Inhibitors for amylases have been found in wheat (**11**) and Leoti sorghum (**8, 12**). In 1953, WEURMAN (**15**) was the first to study a thermolabile inhibitor for pectinase which was precipitated from pear sap with acetone. The maximum inhibition of pectinase, using pectin and pectic acid as substrates, was found to be 82 and 50%, respectively.

Studies on naturally occurring trypsin inhibitors have been recently reviewed (**9**), and a number of sources have been reported, namely, pancreas, soybean, colostrum, lima bean, ovomucoid, blood plasma, and *Ascaris*. All the trypsin inhibitors isolated so far appear to be proteins, and most of them are remarkably stable to mild acid and heat.

Material and methods

INHIBITOR SOURCE.—Scuppernong of the Muscadine group (*Vitis rotundifolia* Michx.) and Sheridan, Concord, Niagara, Portland, and Leutie (all varieties of *Vitis labrusca* L.) were the grapes tested for inhibitor source. Leaves from these six varieties were collected during July, 1956, from the Method Horticultural Station of North Carolina State College. The leaves were in excellent condition, being free from disease and insect damage. Immediately after harvesting, they were thoroughly washed in cool tap water, air-dried to their original weight, and stored in polyethylene freezer bags at − 10° C.

EXTRACTIONS OF WATER-SOLUBLE GRAPE-LEAF INHIBITOR (GLI).—Several procedures were tested for their efficiency in extracting the inhibitor substance. However, the most complete extraction was obtained by use of aqueous extracts from fresh or frozen leaves as described here. Dried preparations were particularly poor for extraction purposes. For example, when leaves were dried for 40 hours at 45°–48° C., an aqueous extract from the dried preparation was about one-third as effective in inhibiting pectinase as was the equivalent amount of fresh material.

In the procedure of choice the extracts were prepared by placing 20–40 gm. of shredded leaves

[1] This study was carried out under a co-operative project with the Department of Horticulture of the North Carolina Agricultural Experiment Station and the U.S. Food Fermentation Laboratory, one of the laboratories of the Southern Utilization Research and Development Division, ARS, USDA.

in a Waring Blendor[2] containing 400 ml. of distilled water and blending for 3 minutes. The grape-leaf slurry was pressed through three thicknesses of cheese cloth, and the liquid was centrifuged for 15 minutes at 3000 r.p.m. The clear, straw-colored extract was preserved with a few drops of toluene and stored at 4° C. Ammonium sulfate and acetone were used in an attempt to fractionate the inhibitor from the water-soluble extracts.

ACETONE EXTRACTION OF INHIBITOR (API).—An acetone powder (3) was prepared from the Scuppernong variety in a freezer cabinet where all the materials and equipment were maintained at approximately − 10° C. In preparation, 25 gm. of frozen leaves were placed in a Waring Blendor with 200 ml. of cold acetone and extracted for 3 minutes. The blended material was then filtered through a Büchner funnel, washed with excess acetone, and the residue air-dried on filter paper at room temperature. The leaf fiber was separated from the powder by using a 40-mesh sieve, and the resulting fine powder was stored in a desiccator.

ENZYME SOURCE.—A crude pectinase solution was prepared from partially dried cucumber (Model variety) flowers still adherent to small cucumbers (1 in. in diam.). The flowers were obtained during the summer of 1955 at a pickling plant located in Ayden, North Carolina. Previous work (4) has demonstrated that such cucumber flowers are a potent source of pectinase resulting chiefly from a heavy growth of mold in the flower.

The pectinase solution was prepared by blending 20 gm. of flowers in 400 ml. of 2% NaCl solution for 3 minutes. The mixture was filtered, and the extract was dialyzed for 3 hours in cellophane tubing against tap water followed by 1 hour against distilled water. The clear, crude pectinase solution from the flowers was preserved with a few drops of toluene and stored at 4° C.

The purified polygalacturonase (PG) was kindly supplied by E. F. JANSEN, Western Utilization Research and Development Division, USDA, Albany, California. It was purified from a commercial preparation of fungal pectinase according to a method described by JANSEN and MACDONNELL (5).

MEASURING ENZYME ACTIVITY AND INHIBITOR.—The viscometric method as described in detail by BELL *et al.* (1) was used because of its sensitivity. Enzyme activity is expressed as the reciprocal of time (hours) to reach a 50% loss in viscosity of a 1.0% sodium polypectate solution, buffered with 0.02 *M* NaOH–citric acid at pH 5.0 and at 30° C. Thus 100 units of pectinase activity are equivalent to 20 hours reaction time or 0.05 reciprocal hours at the above conditions. Standard enzyme-reaction curves were used to convert loss-in-viscosity measurements to units of activity. The influence of the grape-leaf extracts containing the inhibitor was determined by mixing two volumes of a pectinase or PG/enzyme solution with one volume of an inhibitor solution. Controls, using water in place of inhibitor and/or enzyme solution, were included in the tests. For the pectinase enzyme test, 1 ml. of this solution was mixed with 5 ml. of substrate solution.

[2] The mention of firm names or trade products does not imply that they are indorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Results

The presence of a pectinase inhibitor in grape leaves was first demonstrated in aqueous extracts of leaves from the Concord variety. When a very concentrated extract of leaves (19 gm/100 ml of water) was used, it was found that the standard pectinase solution (93 units) was reduced in activity 85%. Further treatments of the leaf extract by dialysis in cellophane tubing against water, or dialysis followed by heating in a water bath to 80° C. for 10 minutes, reduced but did not destroy its inhibitory properties. The dialyzed and the dialyzed-heated extracts reduced the pectinase activity 72 and 58%, respectively.

INFLUENCE OF INHIBITOR CONCENTRATION ON PECTINASE.—Crude, grape-leaf extracts of Concord and Scuppernong varieties, at inhibitor concentration levels covering a hundred-fold range, were tested against a pectinase-flower extract. Small increments of extracts (GL) of the Concord and Scuppernong varieties gave marked reductions in enzyme activity (fig. 1), and the latter variety showed the highest concentration of inhibitor per weight of leaf. When the values for the two curves are plotted using the log of the inhibitor concentrations, fairly straight lines can be drawn through the points representing 20–90% reduction in enzyme activity; this suggests a first-order reaction.

INFLUENCE OF GRAPE VARIETY ON INHIBITOR CONCENTRATION.—The leaves from six varieties of grapes were examined for inhibitor content. Fully mature leaves from each of the varieties were used; immature leaves for one variety (Concord) were also included. These were about half the size of, and lighter in color than, the mature leaves of the same variety.

Information concerning the varieties tested, average weight per leaf, pH value of extract, and percentage reduction in pectinase activity are presented in table 1. The varieties are listed in the decreasing order of inhibitor concentration as measured by reduction in enzyme activity. The Scuppernong variety reduced the enzyme activity 95% and was the most effective of the six varieties examined. Leutie

variety was the least effective, giving a reduction of enzyme activity of 37%. By referring to the curves in figure 1, which show the influence of increasing levels of grape-leaf inhibitor on enzyme activity, it may be computed that the inhibitor content of Scuppernong variety would be about twenty times

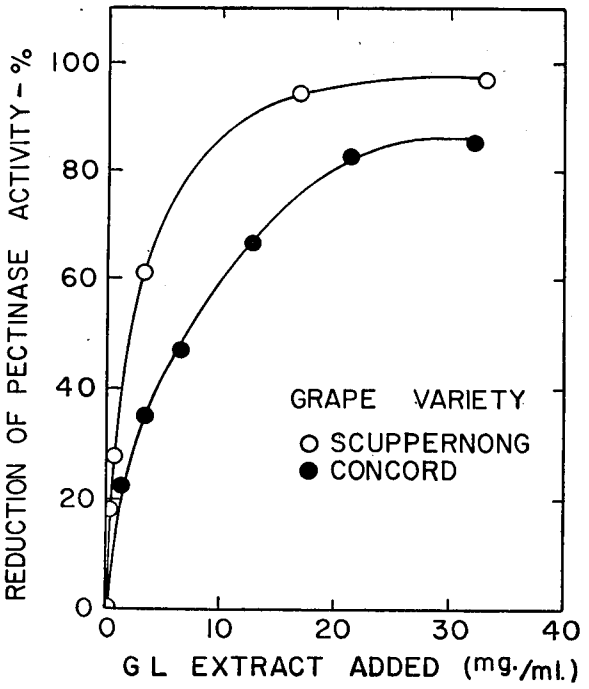

FIG. 1.—Relationship of concentration of grape-leaf (GL) extracts of Concord and Scuppernong grape varieties to reduction of pectinase activity.

TABLE 1

CONCENTRATION OF PECTINASE INHIBITOR IN LEAVES FROM SIX GRAPE VARIETIES

Grape variety	Average fresh weight per leaf (gm.)	Inhibitor extract pH	Pectinase enzyme activity inhibited[a] (%)
Scuppernong	1.0	3.9	95
Sheridan	3.8	4.6	72
Concord	5.0	4.2	64
Niagara	6.2	4.3	57
Portland	4.0	4.5	54
Leutie	5.3	4.4	37
Concord (immature)	2.3	3.5	28

[a] 5% Grape-leaf extracts tested against pectinase solution.

the concentration of Leutie and three times that of Concord. The inhibitor concentrations of the remaining three varieties were either slightly above or below that of Concord. The fully developed leaves from the Concord variety had about ten times more inhibitory activity than the immature leaves of this variety.

INFLUENCE OF DIALYSIS AND ACETONE PRECIPITATION OF WATER-SOLUBLE EXTRACTS AND THEIR STABILITY TO HEAT.—These factors were further investigated with leaves from Scuppernong and Concord varieties; table 2 gives the percentage of pectinase activity inhibited, based on a standard enzyme control. As previously shown (table 1), the Scuppernong inhibitor extract, on a weight basis, was highest in concentration. The aqueous extracts of the grape leaves, with and without dialyzing, were very stable to boiling but less stable to autoclaving. However, the inhibitor in the acetone fraction, as compared to the above extracts, was much less stable to boiling and even less to autoclaving. There was a slight loss in inhibitor content of the aqueous extracts upon dialysis but an appreciable loss in acetone precipitation. A pectinase inhibitor was also present in the acetone-soluble fraction. Further studies are under way on this inhibitor.

TABLE 2

INFLUENCE OF TEMPERATURE, DIALYSIS, AND ACETONE PRECIPITATION ON PECTINASE INHIBITOR FROM SCUPPERNONG AND CONCORD GRAPE LEAVES

Heat treatment[a]	Pectinase activity inhibited[b]: Water-soluble extract (%)	Water-extract dialyzed (%)	Water-soluble extract precipitated with acetone[c] (%)
		Scuppernong	
No heat	98	95	50
Boiled	98	95	13
Autoclaved	91	83	7
		Concord	
No heat	65	51	20
Boiled	62	51	13
Autoclaved	45	35	4

[a] Heat treatment: boiled—brought to rapid boil and cooled; autoclaved —15 lbs pressure/sq in for 15 minutes and cooled. Inhibitor source: 5 gm. of grape leaves per 100 ml.

[b] Pectinase enzyme source: 5 gm. of cucumber flowers per 100 ml.

[c] One volume water-soluble extract treated with three volumes acetone; precipitate dried and redissolved in water. A pectinase inhibitor was also present in the acetone-soluble fraction, and studies on this inhibitor will be reported later.

TREATMENT OF THE GRAPE-LEAF EXTRACT WITH AMMONIUM SULFATE.—To 50-ml. amounts of a water-soluble extract from Scuppernong leaves, 15, 20, 25, and 30 gm. of ammonium sulfate were added, and the precipitates centrifuged out. Each precipitate was dissolved in 50 ml. of distilled water and dialyzed in cellophane tubing for 3 hours against cool, running tap water followed by 1 hour against distilled water. The results for enzyme inhibition with the 15, 20, 25, and 30 gm. ammonium sulfate precipitations were 56, 68, 73, and 95% reduction in pectinase activity, respectively. The highest concentration of ammonium sulfate used (30 gm. in 50 ml. of extract or about 85% saturated) gave the precipitate which caused the highest reduction in enzyme activity. The inhibitor was only partially precipitated by ammonium

sulfate, and there was no sharp break indicating a change in solubility. This method of extraction would seem not to be suitable for further investigations in separation and purification of the inhibitor substance.

ACETONE POWDER FROM GRAPE LEAVES AND INHIBITION OF PURIFIED POLYGALACTURONASE ACTIVITY.—The acetone powder inhibitor (API) was prepared from Scuppernong variety leaves as described earlier. The results for two inhibitor concentrations, along with aqueous controls, tested against three levels of PG are presented in table 3. The three concentrations of PG enzyme, 0.01, 0.05, and 0.10 μgm/ml, used as controls measured 167, 800, and 1850 units of enzyme activity, respectively. By adding 0.2 mg/ml of API, these three activity levels of PG were reduced 22 to 27%. The higher level of API (2.0 mg/ml) gave a higher percentage of reduction for each of the three PG concentrations (57–61%).

For the determination of the type of inhibition, a standard enzyme level (0.05 μgm/ml of PG) and a standard inhibitor level (2 mg/ml of acetone powder) were used against six substrate concentrations ranging from 0.33 to 2.0% (final reaction concentration) and buffered with citrate at pH 5.0 and at 30° C. The data, expressed as the double reciprocal plot according to LINEWEAVER and BURK (10), indicate that the inhibitor in the acetone powder is competitive in nature (fig. 2).

Discussion

The Scuppernong grape is a variety of the Muscadine group (*Vitus rotundifolia* Michx.) and was

TABLE 3

INACTIVATION OF PURIFIED POLYGALACTURONASE (PG) BY GRAPE-LEAF–ACETONE POWDER (AP) INHIBITOR PREPARATION

PURIFIED PG CONCENTRATION (μgm/ml)[a]	AP INHIBITOR CONCENTRATION (mg/ml)[a]	RELATIVE ENZYME ACTIVITY: Pectinase (units)	RELATIVE ENZYME ACTIVITY: Inactivation by AP inhibitor (%)
0.01	0	167	0
.01	0.2	130	22
.01	2.0	72	57
.05	0	800	0
.05	0.2	585	27
.05	2.0	345	57
.10	0	1850	0
.10	0.2	1400	24
0.10	2.0	725	61

[a] Test conducted with 4 ml. of enzyme solution plus 2 ml. of inhibitor solution or water for control.

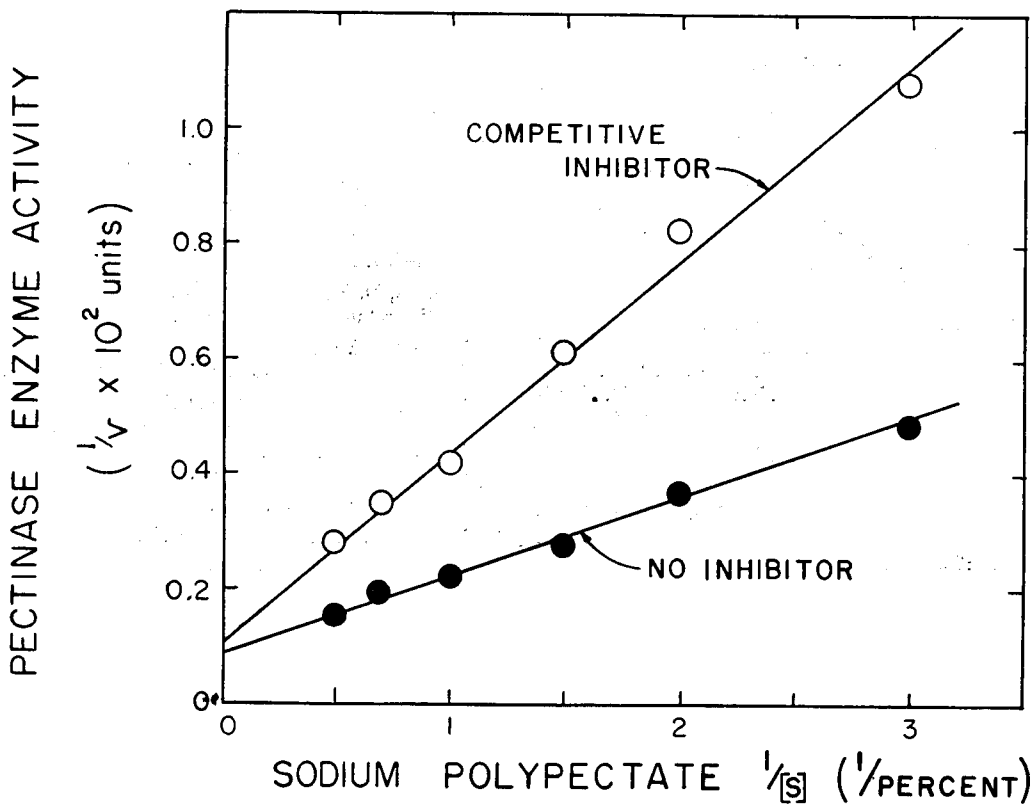

FIG. 2.—Reciprocal of initial velocities ($1/v$) plotted against reciprocal of substrate concentrations ($1/S$) for the reaction with no inhibitor and with inhibitor.

the highest in inhibitor content of six varieties tested. The Muscadine grapes are indigenous to the southern coastal plain area of the United States, and there are a number of additional varieties of this group which should be screened for inhibitor content. DEARING (2) in 1938 contributed a most complete report on the Muscadine grapes, which included history, breeding, culture, and uses.

The results presented suggest that there are two or more pectinase inhibitors present in the material tested and that these active substances are compounds of high molecular weight. Further work on the concentration, purification, and characterization of the inhibitor(s) is now in progress.

Summary

1. A water-soluble substance in grape leaves which inhibits both purified polygalacturonase (PG) and pectinase from mold-laden cucumber flowers is reported.

2. Of the six grape varieties tested, the leaves from the Scuppernong variety of the Muscadine group (*Vitus rotundifolia* Michx.) contained the highest inhibitor content.

3. The inhibiting substance was stable to heat, non-dialyzable through cellophane membrane against running tap water and distilled water, and could not be completely precipitated with acetone or concentrated ammonium sulfate.

4. The reduction in pectinase activity obtained was directly related to the inhibitor concentration used. The reaction between polygalacturonase, the substrate, and the inhibitor (acetone-powder preparation) was that of competitive inhibition.

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LITERATURE CITED

1. BELL, T. A.; ETCHELLS, J. L.; and JONES, I. D. A method for testing cucumber salt-stock brine for softening activity. U.S. Dept. Agr., ARS-72-5. 1955.
2. DEARING, C. Muscadine grapes. U.S. Dept. Agr. Farmers' Bull. no. 1785. Revised. 1947.
3. COLOWICK, S. P., and KAPLAN, N. O. Methods in Enzymology. Vol. 1. Academic Press, Inc., New York. 1955.
4. ETCHELLS, J. L.; BELL, T. A.; and JONES, I. D. Studies on the origin of pectinolytic and cellulolytic enzymes in commercial cucumber fermentations. Food Technol. 9(3): 14, 16. 1955.
5. JANSEN, E. F., and MACDONNELL, L. R. Influence of methoxyl content of pectic substances on the action of polygalacturonase. Arch. Biochem. 8:97–112. 1945.
6. JANSEN, E. F.; MACDONNELL, L. R.; and JANG, R. Simultaneous actions of polygalacturonase and pectinesterase on pectin. Arch. Biochem. 8:113–118. 1945.
7. KERTESZ, Z. I. The Pectic Substances. Interscience Publishers, New York. 1951.
8. KNEEN, E., and SANDSTEDT, R. M. Distribution and general properties of an amylase inhibitor in cereals. Arch. Biochem. 9:235–249. 1946.
9. LASKOWSKI, M., and LASKOWSKI, M., JR. Naturally occurring trypsin inhibitors. Adv. in Protein Chem. 9:203–242. 1954.
10. LINEWEAVER, H., and BURK, D. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56:658–667. 1934.
11. MILITZER, W.; IKEDA, C.; and KNEEN, E. The mode of action of an amylase inhibitor from wheat. Arch. Biochem. 9:321–329. 1946.
12. MILLER, B. S., and KNEEN, E. The amylase inhibitor of Leoti sorghum. Arch. Biochem. 15:251–264. 1947.
13. RAHMAN, M. B., and JOSLYN, M. A. Properties of purified fungal polygalacturonase. Food Res. 18:301–304. 1953.
14. WEIER, T. E., and STOCKING, C. R. Histological changes induced in fruits and vegetables by processing. Adv. in Food Res. 2:297–342. 1949.
15. WEURMAN, C. Pectinase inhibitors in pears. Acta Bot. Neerland. 2:107–121. 1953.
16. WHISTLER, R. L., and SMART, C. L. Polysaccharide Chemistry. Academic Press, Inc., New York. 1953.