CELLULASE INHIBITOR IN GRAPE LEAVES[1]

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Introduction

Cellulose, a linear polymer of β-D-glucopyranose, has been estimated to be the most abundant of all organic substances, embracing about one-third of all plant matter (**21**); the primary structural element is composed largely of cellulose. The walls of parenchyma cells in most edible fruits and vegetables have circular or oblong regions of very thin cellulose layers (**20**). Parenchyma cells are held together by pectic substances, and any food-processing method that reacts on either pectic or cellulosic substances will have an effect on the finished product, usually resulting in a softer product of less desirable texture.

In a recent paper, BELL and ETCHELLS (**2**) reported a pectinase enzyme-inhibitor in grape leaves. The present investigation deals with a water-soluble, thermostable, high-molecular-weight substance, also found in grape leaves, which inhibits the enzymatic hydrolysis of soluble cellulose.

An attempt to control softening-type spoilage in the commercial salting of cucumbers prompted these studies. Enzymatic (**7**) and microbial (**8**) investigations into the problem have shown that pectinase and cellulase were associated with cucumber salt-stock softening and that these enzymes were introduced into the brine through the growth of higher fungi in the cucumber flowers prior to their introduction to the brining tanks. The principal species of fungi, isolated from the growing cucumber which included the small fruit and attached flower, were potent sources of pectinase and cellulase (**8**). Cucumber flowers, still adhering to the small fruit and collected at commercial brining stations in different parts of the country, were also good sources of both hydrolytic enzymes (**3**). Application of the pectinase and cellulase inhibitors from grape leaves was made in a study (**9**) with small-scale cucumber fermentations; increasing levels of grape-leaf extract gave increasing firmness to the cucumbers that had been treated with cucumber flowers as a source of both enzymes.

In 1956, REESE (**15**) reviewed and evaluated the enzymatic hydrolysis of cellulose. The complete breakdown of unaltered cellulose by cell-free filtrates from the growth of highly active cellulolytic organisms, such as *Myrothecium verrucaria*, has not been clearly demonstrated. Investigators (**14, 16, 18**) are not in agreement as to whether hydrolysis of cellulose to cellobiose is unienzymatic or multienzymatic or even whether the reactions are completely enzymatic. Most, if not all, enzyme studies (**15**) have been conducted with modified, native cellulose converted to linear cellulose chains by the use of such agents as strong acids (72% sulfuric or 85% phosphoric), strong alkali, or mechanical disintegration.

The sodium salt of carboxymethyl cellulose (CMC) with a degree of substitution (DS) of less than one was recommended by LEVINSON and REESE (**12**) for the substrate in measurements of cellulase activity, and several investigators (**1, 3, 4, 11, 18, 22, 23**) have measured cellulase activity with this type of substrate. The enzymatic hydrolysis of CMC cleaves the 1-4-β-glucosidic linkages with the formation of reducing sugars and with a rapid loss in viscosity. The term "cellulase" in this paper is a measurement of such a reaction and is considered synonymous with "cellulase Cx" (**17**).

The in vivo cellulose-decomposing fungi and the toxic antifungal agents of particular interest to the textile industry have been given considerable attention by SIU (**19**). Only a limited number of investigators has dealt with cellulase inhibitors. ZIESE (**24**)

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reported that snail cellulase in a phosphate buffer was inhibited by increasing concentrations of cysteine and glutathione. Using cell-free extracts of sheep-rumen micro-organisms as a cellulase source, FESTENSTEIN (10) reported that glucono-lactone inhibited the hydrolysis of CMC at a constant value of 60% when the glucono-lactone concentration was sufficient to inhibit cellobiase completely. BASU and WHITAKER (1) in 1953 surveyed a large number of potential inhibitors which included salts of heavy metals, oxidizing and reducing agents, acidic and basic dyes, neutral salts, and thiol-reacting agents (iodoacetate and *p*-chloromercuribenzoate). They assayed these agents against cell-free filtrates of *M. verrucaria* as the cellulase source and modified cellulose as the substrate. Both stimulation and inhibition of the enzyme by different agents were reported; the pH and concentration of the agent tested were most important in determining whether inhibition or stimulation occurred. From the reactions of the thiol agents these workers suggested that thiol groups were essential for cellulase activity (1). This has been confirmed by SISON *et al.* (18) with a purified cellulase preparation from the wood-destroying fungus, *Poria vaillantii*.

Naturally occurring inhibitors for cellulase have not been reported, although inhibitors for certain hydrolytic enzymes, such as pectinase inhibitor from pears and grape leaves, amylase inhibitor from wheat and Leoti sorghum, and trypsin inhibitor from soybean and pancreas, have been studied. These studies were reviewed briefly in an earlier paper (2).

Material and methods

INHIBITOR SOURCE.—Mature grape leaves from the following varieties were tested for cellulase inhibition: Concord (*Vitis labrusca* L.) and varieties of the Muscadine group (*V. rotundifolia* Michx.)—Scuppernong, Thomas, Topsail, Creswell, Willard, Burgaw, and an unknown "wild" strain. The Muscadine grapes are native to the southeastern coastal area of the country; their culture has been studied extensively (6). Leaves from the test varieties were collected during the summers of 1956 and 1957 from the Method Horticultural Station of North Carolina State College and from the Coastal Plain Experiment Station, Willard, North Carolina. The fresh leaves, free from insect and disease damage, were washed in tap water, spread out on cloth to air dry, then stored in polyethylene freezer bags at −10° C.

EXTRACTION OF WATER-SOLUBLE GRAPE-LEAF INHIBITOR.—The inhibitor extracts were prepared by placing 20–40 gm. of shredded leaves in a Waring Blendor containing 400 ml. of distilled water and blending for 3 minutes. The slurry was pressed through three thicknesses of cheesecloth, and the water-soluble extract was centrifuged for 15 minutes at 3000 r.p.m. The clear, straw-colored extracts were stored with a few drops of toluene at 4° C. Where extraction procedures were modified, the methods are stated later.

ENZYME SOURCES.—Cucumber-flower (CF) cellulase was prepared from partially dried cucumber flowers (Model variety), adhering to small-sized cucumbers (1–1⅛ in. diameter). These flowers were collected during the 1955 season at a pickle plant in Ayden, North Carolina, and stored in polyethylene bags at −10° C. Later, CF cellulase solutions were prepared by blending 20-gm. amounts of flowers in 400 ml. of 2% NaCl solution for 3 minutes. Each slurry was filtered through double thicknesses of cheese cloth, centrifuged for 20 minutes at 3000 r.p.m., and dialyzed in cellophane tubing against water for 4 hours. The CF-cellulase solutions were stored for short periods with a few drops of toluene at 4° C.

Four commercial cellulases were also used: enzymes 19AP, 35, and 36, supplied by Rohm and Haas Company, Philadelphia, Pennsylvania, and enzyme XT-541 supplied by Miles Chemical Company, Clifton, New Jersey.

MEASURING ENZYME ACTIVITY AND INHIBITION.—The viscometric method described by BELL *et al.* (4) was used for measurements. Enzyme activity is expressed in cellulase units; 100 units equal 50% loss in viscosity in 20 hours of a 1.0% CMC-7M solution (Hercules Powder Company, Wilmington, Delaware), buffered at pH 5.0 (1.33 gm. NaOH and 3.53 gm. citric acid/l H_2O), and at 30° C. Standard enzyme-reaction curves were used to convert percentage loss in viscosity to units of activity. Inhibitor tests were made by mixing two parts enzyme with one part inhibitor solution; water controls were used in place of inhibitor and/or enzyme solutions. When grape-leaf extracts were treated with chemical agents, the extracts were dialyzed and adjusted to pH 5 prior to enzyme reaction.

Results and discussion

The presence of a cellulase inhibitor was first demonstrated in water extracts of leaves from the Concord variety of grape. In preliminary experiments very concentrated extracts (19 gm/100 ml) from these leaves inhibited 360 units of CF cellulase by 95%. Further studies indicated that the inhibitor present in the leaf extract was relatively stable to heat and was non-dialyzable. For example, the extract, after dialysis against water for 4 hours using cellophane as the dialyzing membrane, retained 91% of its inhibitory activity; heating the dialyzed extract to a temperature of 80° C. for 10 minutes in a water

bath resulted in a loss of only 12% of its inhibitory activity.

INHIBITOR CONCENTRATIONS AND ENZYME SOURCE.—Leaves of Scuppernong grape (Muscadine group—*Vitis rotundifolia* Michx.) were found to be about five times higher in inhibitor concentration than leaves of the Concord variety (*Vitis labrusca* L.). With this information, seven varieties of the Muscadine group (Burgaw, Creswell, Scuppernong, Thomas, Topsail, Willard, and a "wild" strain) were assayed against two cellulase enzymes, 19AP (300 units/ml) and CF (350 units/ml). The 5% aqueous extracts of these leaf samples inhibited CF cellulase 98–99% and 19AP cellulase 50–70%. Since there was no apparent difference in inhibitor concentrations of the seven varieties, Scuppernong was chosen for further studies and the extract designated GLI (grape-leaf inhibitor).

Small increments of GLI extract gave marked reduction in cellulase activity for CF and 19AP enzymes (fig. 1). The CF cellulase was much more sensitive to inhibition than was 19AP. A plot of the logarithm of GLI concentration (mg/ml of GLI added) against percentage reduction of cellulase activity gave a straight line, thus suggesting a first-order reaction. It was concluded that 19AP and CF cellulases are different so far as the inhibitory effects of GLI are concerned. Similar observations have been made by other investigators: glucono-lactone inhibition of rumen cellulase activity by 60% (**10**), differences in heat stability of several fungal cellulases (**34, 24**), and multi-enzyme systems (**14, 23**).

Data are presented in table 1 for five sources of cellulase as to differences in heat stability and in inhibition by the GLI extracts. CF cellulase was inactivated by GLI and by heat treatments of 75° C. and 100° C. Enzymes 19AP, 36, and XT 541 were more resistant to heat; 75° C. for 20 minutes reduced the activity of XT 541 cellulase by only 8%, 19AP by 26%, and 36 by 48%. The four commercial preparations were not completely inhibited by 100° C. for 20 minutes; enzyme 35 was the most sensitive to both heat and GLI treatments of the four and corresponded closely with CF cellulase (table 1). Further inhibition was noticed when GLI was added to the heat-stable fractions of the commercial cellulases—77–94% cellulase inhibition was obtained (table 1).

The data clearly show that the inhibition of cellulase by GLI is dependent upon source of the enzyme. Furthermore, it will be noted that those cellulases relatively unaffected by either heat or

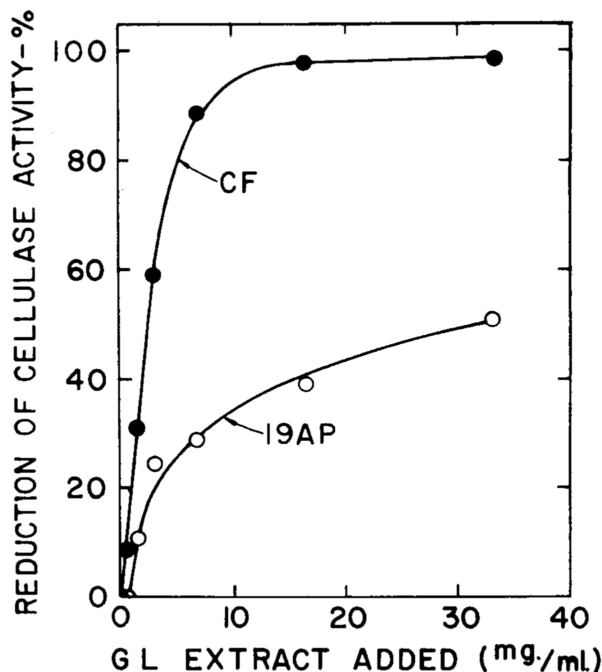

FIG. 1.—Relationship of concentration of leaf (GL) extract of Scuppernong grape to reduction of cucumber-flower (CF) and 19AP cellulases.

TABLE 1

EFFECT OF TEMPERATURE AND GRAPE-LEAF INHIBITOR ON THE ACTIVITY OF CUCUMBER-FLOWER CELLULASE AND FOUR COMMERCIAL CELLULASES

		Cellulase activity inhibited by			
			Heat		
Enzyme source	Cellulase activity[a] (units)	Grape-leaf inhibitor[b] (%)	75° C. for 20 min. (%)	100° C. for 20 min. (%)	Heat (100° C. for 20 min.) plus GL inhibitor (%)
CF........	370	98	98	100	..
35........	312	96	80	77	94
36........	326	61	48	59	88
19AP......	465	58	26	55	80
XT 541....	455	44	8	46	77

[a] Cellulase concentration: 5% CF extract; 0.1 mg/ml of each of four commercial enzymes: 19AP, 35, 36, and XT 541.

[b] 10% water extract of Scuppernong variety grape leaves, dialyzed.

GLI were rendered less active when heat and GLI were used together. Results of electrophoretic and chromatographic investigations (**14, 23**) with fungal cellulases, including 19AP and *Myrothecium verrucaria*, have shown multiple-enzyme components. The differences in sensitivity of the five enzyme sources to heat and GLI (table 1) may be the result of such components, their properties, and their concentrations.

Properties of the GLI extracts

Solubility in water.—Water extracts of grape leaves were adjusted to pH levels of 1.5, 3.8, 5.0, 8.0, 9.0, 11.0, and 11.6 with either 1 *N* NaOH or 1 *N* HCl. The treated extracts were centrifuged at 3000 r.p.m. for 20 minutes and the supernatants and precipitates assayed at pH 5 for the cellulase inhibitor. Above pH 8, the inhibitor gradually precipitated out of solution until the pH value reached 11.6, where it was again soluble. A slight precipitation and little or no destruction of the inhibitor resulted at pH 1.5. The inhibitor was most soluble at pH 3.8 and 5.0.

Solubility in acetone and ethyl alcohol.—With four parts acetone to one part of GLI water-extract and pH values adjusted to 1.5–11.6, as with the above water-solubility tests, the inhibitor was found in the acetone fractions at the acid pH values and was precipitated at the alkaline values. With 95% ethyl alcohol in the same ratio, four to one, the inhibitor was soluble in the acid and precipitated in the alkaline pH values studied.

Solubility in dietyhl ether.—Ether was bubbled through the GLI extract, using a reflux condenser, and the ether-soluble fraction collected in a side flask. The inhibitor was absent in the ether fraction (ether evaporated and soluble material taken up in water) and remained in the water extract.

Efect of heat.—GLI extracts were extremely resistant to inactivation by heat. Water-extracts of grape leaves (5%) were exposed for 2, 4, 8, 16, and 32 minutes in a boiling water bath and to 15 minutes of autoclaving at 15 lb/sq in. There were very small losses in activity following these heat treatments. To test the influence of pH and heat, GLI extracts were adjusted to pH 5.0 and pH 9.0 and heated to 90° C.; these treatments did not inactivate the substance.

Inhibitor activity of dried and ashed grape leaves.—Scuppernong grape leaves, dried for 40 hours in an air-flow oven at 45°–48° C., were extracted in water in the usual manner and assayed, at the same time as a comparable weight of an aqueous extract of fresh Scuppernong grape leaves, against CF cellulase. The dried leaves had lost more than half their inhibitor activity. Complete loss of inhibitor activity was encountered when the leaves were ashed in a muffle furnace and the mineral constituents, along with an equivalent weight of grape leaves, tested against CF and 19AP cellulases. These results indicate that the inhibitor is heat labile when present in the leaf during air-flow drying at 45°–48° C. and that the inhibitory effect of the extract can be ascribed not to its mineral constituents but to its organic nature.

Effect of dialysis.—GLI extracts (100 ml.) were placed in cellophane and collodion bags and dialyzed against 1500 ml. each of distilled water and of two buffer solutions—0.005 *M* K_2HPO_4 (pH 7.9) and 0.03 *M* KH_2PO_4 (pH 4.6). The dialyzing solutions were changed every 4 hours for 3 days except during the nights. The dialyzates of each solution were concentrated under reduced pressure to approximately 100 ml. and cellulase-inhibitor assays determined on this concentrate and on the dialyzed extract. Only small traces of inhibitor, as tested against 19AP were detected in the three dialyzates. The cellulase inhibitor was not dialyzable under these conditions, and it is concluded that the inhibitor is of relatively high molecular weight, probably more than 10,000.

Adsorption of inhibitor.—The cellulase inhibitor was completely adsorbed on carbon black (10 gm. Norite per 100 ml. GLI extract) by direct shaking in a flask for 10 minutes. The inhibitor was not eluted with 1 *N* acetic acid, slightly eluted with 1 *N* NaOH, and completely eluted with acetone. The use of chromatographic columns (**5**), Amberlite IRA-400 and Dowex-1, each charged with 0.1 *M* sodium borate, completely adsorbed the inhibitor on each column. Continuous flow of sodium borate solution, followed by 1 *N* HCl, did not elute the inhibitor. A third mixed-resin column was tried which was prepared from IR-120 (H^+) and IRA-400 (CO_3^{--}). The cellulase inhibitor passed through this column unadsorbed. These column studies indicate that the cellulase inhibitor was not a carbohydrate.

Studies on chemical nature of inhibitor

Trichloroacetic acid (TCA).—The GLI extracts containing 2.6% TCA were held at room temperature (about 23° C.) and at 100° C. for 5 minutes. The precipitates from the two treatments were filtered and the TCA removed from the four fractions by ether extraction, and then the fractions were tested against 19AP cellulase for inhibition. The inhibitor was found only in the supernatant fraction for the 23° C. treatment and in both fractions, precipitate and supernatant, for the 100° C. treatment.

Urea.—Urea is frequently used as an analytical

tool because of its ability to rupture a number of hydrogen bonds of the secondary structure of protein and because of its protein-dissolving effect. Accordingly, urea was added to GLI extract until an 8 *M* solution was obtained. This treatment had no effect upon the cellulase inhibitor.

ACETONE.—Extraction of frozen grape leaves at about −10° C. (**2**) with 100% and 50% acetone gave acetone powders and soluble fractions which inhibited enzyme activity of 19AP cellulase. The soluble fractions contained the highest inhibitor activity for both treatments, and the acetone powder had very little.

AMMONIUM SULFATE.—Increasing the concentration of $(NH_4)_2SO_4$ in GLI extracts by 25%, 50%, 75%, and 100% of saturation, and measuring 19AP cellulase inhibitor activity of each precipitate and supernatant fraction, produced no clear-cut fractionations. The salted-out precipitates, when redissolved in water and dialyzed, exerted no inhibitory effect upon cellulase activity; the soluble fractions, when dialyzed free of $(NH_4)SO_4$, possessed cellulase inhibitor activity.

PROTEOLYTIC DIGESTION.—GLI extracts were treated with pepsin, papain, and trypsin, each at 0.0025% concentration, for 24 hours at 40° C. The cellulase inhibitor was found in each proteolytic digest treatment to be stable in the soluble fractions and not present in the precipitates. The proteolytic enzymes did not affect the inhibitor activity.

The effects of the various agents (TCA, urea, acetone, $(NH_4)_2SO_4$, and proteolytic enzymes) on the GLI extracts lead to the conclusion that the principal substance in grape leaves which inhibits cellulase activity is not a protein.

COMPARISON OF GLI WITH AN AMYLASE INHIBITOR

Some of the properties of an amylase inhibitor extracted from Leoti sorghum by MILLER and KNEEN (**13**) are similar to those described for the cellulase inhibitor reported in this study. These properties are: (*a*) extreme resistance to inactivation by heat; (*b*) non-dialyzability in tap water, indicating a high molecular weight; (*c*) solubility in water, acetone, and ethyl alcohol below pH 7, precipitated above pH 7, the reactions reversible; (*d*) insolubility in diethyl ether; (*e*) non-protein nature suggested by precipitation test with ammonium sulfate; and (*f*) adsorption on carbon black complete with elution by weak solution of sodium hydroxide or acetone.

To test the amylase inhibitor against cellulase, a sample of Leoti sorghum was obtained from Dr. J. A. JOHNSON of Kansas State University, Manhattan, Kansas. A 10% water extract was prepared as described (**13**) and tested against 19AP and CF cellulases. The Leoti sorghum extract had no inhibitory effect against 19AP cellulase and only a slight one against CF cellulase. Thus, it is concluded that the two inhibitors are different and are apparently selective to the two enzyme-substrate systems.

MILLER and KNEEN (**13**) suggested that their amylase inhibitor was an organic acid with high molecular weight, containing the indole group and no amino nitrogen. The cellulase inhibitor is a thermostable organic constitutent with high molecular weight, possibly a phenolic compound.

Further studies on the identity, concentration, and purification of the cellulase inhibitor and also of the pectinase inhibitor (**2**), from grape leaves are being made by investigators at the Eastern Regional Research and Development Division, U.S. Department of Agriculture, Philadelphia, Pennsylvania, in co-operation with our laboratory.

Summary

1. A water-soluble substance in grape leaves which inhibits the enzymatic hydrolysis of soluble cellulose is reported. The mature leaves of six grape varieties of the Muscadine group (*Vitis rotundifolia* Michx.) were found to be good sources of the inhibitor.

2. The reduction in cellulase activity obtained was directly related to the concentration of inhibitor used. Cucumber-flower cellulase was more sensitive to inhibition by the grape-leaf substance than were the four commercial cellulases studied.

3. The grape-leaf extracts containing the inhibiting substance were stable to heat, to weak acid and alkali, and to such protein precipitating agents as trichloroacetic acid. The inhibitor was non-dialyzable through cellophane or collodion membranes against water or weakly buffered solutions (3 days).

4. The cellulase-inhibiting substance is not related in structure to carbohydrates or proteins and appears to be a high-molecular-weight organic constituent.

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