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Inhibition of Pectinolytic and Cellulolytic Enzymes in Cucumber Fermentations by Scuppernong Grape Leaves *

John L. Etchells and
Thomas A. Bell

U. S. Food Fermentation Laboratory^b
and

Carlos F. Williams

Department of Horticulture, North
Carolina Agricultural Experiment Sta-
tion, Raleigh, North Carolina

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THE ANNUAL CROP of pickling cucumbers in this country from 1954 to 1956 has been reported (4) at 12 to 14 million bushels with a farm value of more than 21 million dollars to an estimated 50,000 growers. The pickle industry itself has more than doubled in the past 20 years and it is becoming increasingly important that the processing of this crop, worth over 165 million dollars at the manufacturers level, be eventually placed under controlled conditions with a minimum of loss due to spoilage.

One of the spoilage problems that has confronted the industry for many years is that of enzymatic softening of the cucumbers during brine fermentation and storage. It has been estimated (8) that this type of spoilage represents an annual loss of about one million dollars, particularly where the salt-stock cucumbers have deteriorated in firmness to the point where they have to be discarded or at best used in low-quality, low-priced products.

The nature of salt-stock softening under commercial conditions in the South and Southwest has received considerable investigation (2, 3, 6, 7, 8). Results demonstrate that the softening is the result of pectinolytic and cellulolytic enzyme activity in the brine and that these enzyme systems are introduced into the curing vats chiefly by way of the partially dried cucumber flowers that remain attached to the cucumber fruit. In laboratory tests (2) purified polygalacturonase has been demonstrated to soften salt-stock; however, the exact role of the cellulolytic enzyme in the brines has not been clearly established.

Microbial studies strongly indicate that the enzyme activity is chiefly the result of growth by higher fungi (molds) in the cucumber flowers prior to their reaching the brining station. The principal species of molds on the growing cucumber plant have been isolated and studied as to their biochemical properties (7). As a group, most of them are a potent source of various hydrolytic enzymes which cause a break-down of pectic and cellulosic substances.

Several approaches directed toward eliminating or

significantly reducing the concentration of softening enzymes in commercial cucumber brines have been suggested (3, 8). These are: (a) mechanical removal of flowers from the cucumbers; (b) development of new cucumber varieties with a minimum of retained flowers; (c) development of draining procedures to reduce enzyme content of brines before it can affect cucumber firmness; and, (d) inactivation of the enzymes in the brine with specific, non-toxic inhibitors. The draining procedure has received rather thorough investigation (8) and has been widely accepted in southern brining areas.

The use of specific, naturally occurring, non-toxic inhibitors is the approach considered in the present study to control enzymatic softening of brined cucumbers.

In 1953, Weurman (17) reported the first naturally occurring thermolabile inhibitor for pectinase in several varieties of pears; also, he was able to precipitate the active substance from the pear sap with acetone. A number of naturally occurring inhibitors have been reported for other hydrolytic enzymes, such as the amylase inhibitors from wheat and Leoti sorghum (11, 15, 16), and the trypsin inhibitors from soybean, lima bean, colostrum, and pancreas (12). Bell and Etchells (1) have recently found that pectinolytic and cellulolytic enzymes are inhibited by a water soluble fraction of grape leaves. Among the 6 varieties of grapes studied, the leaves of a native American variety—namely, Scuppernong of the Muscadine group (*Vitis rotundifolia Michx.*)—gave the highest inhibitor concentration per gram of leaves.

Use of grape leaves in making fermented dill pickles for home use was suggested by LeFevre (13) in 1927. He states: "It is a good plan to place over the top a layer of grape leaves. In fact, it would be well to place these at both the bottom and top. They make a very suitable covering and have a greening effect on the pickles." The use of grape leaves in cucumber pickles has been handed down from one generation to another in home pickling procedures and may be found in very old recipe books. However, the true value of grape leaves has not been demonstrated in the scientific literature.

MATERIALS AND METHODS

In the present work, Scuppernong grape leaves were used as

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^b One of the laboratories of Southern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

TABLE 1

Treatments employed in the small-scale cucumber fermentations with added cucumber flowers and grape leaf inhibitor (GLI)

Treatment ¹	Cucumber flowers added per barrel	GLI added per barrel
	pounds	pounds
A.....	0	0
B.....	1.25	0
C.....	1.25	0.25
D.....	1.25	0.75
E.....	1.25	1.25
F.....	0	1.25

¹ Each treatment consisted of duplicate 215 pound lots of no. 1 size Model variety cucumbers brined in 45-gallon barrels. The basic brining treatment, 25° sal. (6.6% salt) raised 5° sal. (1.3%) per week to a holding strength of 60° sal. (15.8%), represented commercial practice at the plant where the experimental work was done.

All samples were stored at about 5° C. until they were shipped by air to the laboratory for analysis. The experimental brining work was done during the 1956 season at a commercial pickling plant located in northeastern Texas.

Commercial pectinase experiment. An exploratory experiment was conducted later on during the season in which a commercial pectinase,^{c,d} rather than the cucumber flowers, was used as the source of the enzyme. In this experiment there were 4 treatments in duplicate 45 gal. barrels; these were as follows: Treatment N, control, without enzyme or GLI; Treatment O, 8 g. of pectinase only; Treatment P, 8 g. of pectinase plus 0.25 lb. GLI; and, Treatment Q, 8 g. of pectinase plus 0.75 lb. GLI. The salting procedure, brine sampling and other details were the same as for the first experiment.

Brine analysis. Pectinolytic and cellulolytic enzyme activities of the brine samples were measured by the viscometric method described by Bell *et al.* (3). In this procedure, 100 units of pectinolytic activity equal 50% loss in viscosity in 20 hours of 1.0% sodium polypectate solution in a citrate buffer at pH 5.0, at 30° C. The cellulolytic activity units are equal to same conditions as above except 1.0% sodium carboxymethylcellulose was used as the substrate. Total acidity was measured using 10 ml. aliquots of the brine samples and titrating with 0.111 N NaOH, using phenolphthalein as the indicator. The acidity is expressed as grams of lactic acid per 100 ml. sample. Brine pH was measured with the Beckman^e pH meter, Model H-2. Optical density of the brines was taken with Lumetron^e colorimeter using a 650 m μ filter and 18 mm. diameter test tubes.

The cured salt-stock was judged by personnel of the pickle company for color and acceptability for commercial use; this procedure was essentially that described by Jones *et al.* (10) except the quality categories were increased from 3 (*not acceptable, barely acceptable, and acceptable*) to 5 (*not acceptable, poor, fair, good, and excellent*). Later, the stock was manufactured into sweet pickles and processed dill pickles. These were judged for *appearance, flavor, texture, and overall acceptability*. Each of these 4 pickle characteristics was scored as to one of the following ratings: *not acceptable, poor, fair, good, and excellent*. The U.S.D.A. Fruit Pressure Tester (14) was used for determining the firmness of the salt-stock (3).

RESULTS

Effect of GLI on pectinolytic and cellulolytic enzymes. Pectinolytic and cellulolytic activities of the brine samples at the maximum level (24 hr. period) and the percentage of enzyme inactivation by GLI are presented in Table 2. Maximum enzyme activities in all brines were found 24 hours after the cucumbers were covered with brine. These results are in general agreement with studies by Etchells *et al.* (7) on the maximum enzyme level reached in flower-added brines after a similar time interval. However, the pectinolytic activity of the cucumber flowers used in the present study was much lower than those in the above report. Due to the conditions of the experiment, it was not possible to measure enzyme activity of the cucumber flowers at the plant prior to starting the tests

inhibitor source for the softening enzymes; this inhibitor is referred to throughout as GLI. The effect of this material on small-scale, experimental cucumber fermentations was followed for: (a) enzyme activity; (b) brine acidity and pH; (c) microbial activity during fermentation as indicated by optical density of the brine; and, (d) firmness and general acceptability of the cucumbers after approximately 90 days of fermentation and curing in brine.

The enzyme systems measured in this paper were (a) polygalacturonase (syn. pectinase), which hydrolyzes the glycosidic bonds of pectic acid with simultaneous loss in viscosity and increase in reducing groups; and (b) cellulase (Cx) which hydrolyzes the glycosidic bonds of a soluble cellulose derivative (sodium carboxymethyl-cellulose) with loss in viscosity and an increase in reducing groups. These two systems will be referred to as "pectinolytic activity" and "cellulolytic activity."

Grape leaf inhibitor (GLI). Approximately 20 pounds of Scuppernon leaves were picked at the Coastal Plain Experiment Station, Willard, North Carolina. The leaves were cooled to approximately 5° C. within 2 hours after picking, and held at this temperature overnight. After washing twice in cool tap water, they were air dried to approximately their original weight. Two lots of 500 g. each were placed in an air flow oven at 45-48° C. for 40 hours, then ground in a Wiley mill using 0.5 mm. sieve and stored as a powder at room temperature. The remaining leaves were placed in large polyethylene freezerbags, sealed, and stored at -10° C. Later, the frozen leaves were shipped with dry-ice to the pickle plant just prior to use in the cucumber-fermentation studies.

Source of cucumbers and cucumber flowers. Eighty bushels of No. 1 size (1 in. diameter) Model variety cucumbers, representing 20 bushels from each of four receiving stations, were de-flowered by hand. Additional flowers were obtained from the grading machines at the same four receiving stations. Flowers were separated from sand, grit, and other extraneous material by the use of hardware cloth wire screens, followed by picking over by hand. In all, about 15 pounds of flowers were obtained and in order to assure uniform enzyme concentration these were thoroughly mixed by tumbling in large boxes.

Brining procedure and sampling. Well coopered, clean 45-gallon barrels were used as containers for the brining experiments. The containers were well marked as to treatment and 215 lbs. of de-flowered cucumbers, representing a composite of the stock from the 4 receiving stations, were placed in each.

In the treatments employed (Table 1), flowers and/or GLI were added according to the design of the experiment. The GLI was added as a crude, brine-extract at the time the cucumbers were covered with brine. The extract was prepared by first chopping 2 ounces of grape leaves with a cleaver on a cutting board and then blending the minced leaves in 1 pint of 25° salometer brine (6.6% salt by wt.) with a Waring blender.^c In this manner, the 7 lbs. of fresh leaves were prepared in 2 hours using 2 blenders.

Flowers were equally distributed as the cucumbers were added to the barrels. Next, each barrel was fitted with a "false" wooden head which covered the cucumbers and then about 20 gallons of 25° sal. brine were added. This amount brought the brine level to about 2 inches above the head and about the same distance from the top of the barrel. Twelve pounds of dry salt were added on the head of each barrel and the following day the brines tested 25° sal. ($\pm 2^\circ$). This concentration was increased 5° sal. per week to a holding strength of 60° sal.

Barrels were stored under sheltered conditions and received constant attention throughout the 90-day curing period to preclude active surface yeast development. Brine temperatures during the active fermentation period were in the range of 28 to 30° C.

Barrels were sampled with the equipment described by Etchells and Jones (9) at the following fermentation periods: after 0, 1/4, 1/2, 1, 2, 3, 5, 10 and 30 days. Brine samples were collected in 3-ounce bottles supplied with tin-lined caps and 5 drops of toluene were added to each sample as a preservative.

^c Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

^d Pectinase 46 AP (No. 32) was supplied by Rohm and Haas Company, Philadelphia, Pennsylvania.

and thus forecast the approximate activity of softening enzymes in the brines. It is important to mention that the enzyme activity of cucumber flowers from No. 1 size stock may vary greatly not only between seasons but also for given periods within the same season.

The 3 control treatments, A (cucumbers only), B (cucumbers plus flowers), and F (cucumbers plus GLI) reached levels of pectinolytic activity in the brine of 13, 35, and 2 units respectively. In comparing treatments A and B, the enzyme activity contributed by the cucumbers represented about one-third of the total enzyme activity found; in Treatment F (cucumbers plus GLI) the activity was 2 units as compared with 13 units for A (cucumbers only). This represented an 85% reduction in pectinolytic activity caused by the addition of GLI. In treatments C, D, and E, with 0.25, 0.75, and 1.25 pounds of GLI added per barrel respectively, the pectinolytic enzyme activity levels were reduced from 35 units (enzyme control) to 20, 10 and 6 units or 43, 71 and 83%.

The results for inhibition of cellulolytic enzyme activity in the different treatments followed very closely those discussed for pectinolytic activity (Table 2). The total cellulolytic enzyme concentration provided by the flowers and cucumbers, as shown by the maximum level at the 24-hour period, was in the same range (200 units) as reported in earlier studies (7). In the 3 control treatments, the cucumbers alone contributed 22 cellulolytic units (A), the cucumbers plus flowers 240 units (B), and the cucumbers plus GLI 5 units (F). The cucumbers added less than 10% of the total enzyme activity to the cucumber flower treatments, and the GLI reduced this activity about fourfold. In treatments C, D and E, with 0.25, 0.75, and 1.25 pounds of GLI added per barrel respectively, the cellulolytic enzyme levels were reduced from 240 units (enzyme control) to 172, 118, and 63 units. The percentages of inactivation caused by these three levels of inhibitor were 28, 51, and 74% respectively.

Figure 1 presents the pectinolytic and cellulolytic activity levels during the first 5 days of fermentation for the 1.25 pound GLI treatment (E) as compared to the enzyme control without inhibitor (B). The reduction of activity of both enzyme systems by GLI was very pronounced in these cucumber fermentations.

Firmness of salt-stock. An important objective of these inhibitor treatments was to correlate, if possible, the reduction of softening enzyme activity with improved firmness of cured brine-stock cucumbers. In considering treatments A (no flowers or inhibitor added) and B (flowers only added), the addition of flowers to the fermentation reduced the firmness as measured by the U.S.D.A. pressure tester, from 19 to 13 pounds, a loss of 32%. When the three increasing levels of GLI (C, D, E) were added to the cucumbers plus flower treatment, the per cent loss in firmness decreased (26, 11, and 0%). The highest inhibitor level (E, 1.25 pounds of GLI), gave salt-stock testing 19 pounds; this was equal in firmness to the control treatment (A) with no flowers or inhibitor added.

The influence of inhibitor concentration on relative enzyme (pectinolytic and cellulolytic) activities in the brines at the 24-hour observation period in relation to firmness of the cured salt-stock cucumbers is presented in Figure 2. As the inhibitor

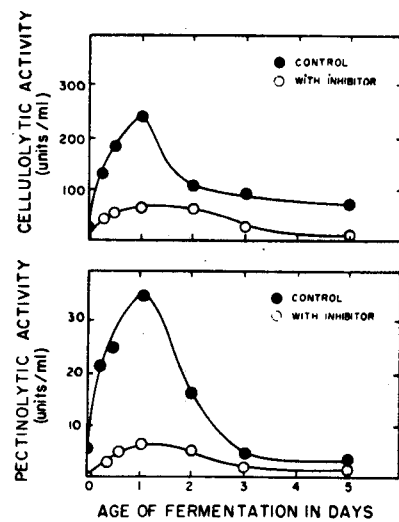


Figure 1. Effect of grape leaf inhibitor (1.25 lbs./bbl.) on pectinolytic and cellulolytic enzyme activity in cucumber brines.

concentration was increased, the relative pectinolytic and cellulolytic enzyme activities decreased and the firmness of the cured salt-stock increased. The firmness of the stock was found to be in direct relationship to inhibitor concentration and inversely related to the activity of the two enzyme systems.

Salt-stock and pickle quality. A panel of two experienced salt-stock judges evaluated coded, 50-pound lots of cured material from the experimental fermentations. Treatments receiving increasing levels of GLI (C, D, and E) were rated *good* to *excellent* as to *acceptability for commercial use*. This was the same rating given salt-stock from Control Treatments A and B. The 32% loss in firmness of Treatment B stock was not sufficient to influence the judges' ratings; experience has shown that an approximate loss of 50% is required to be readily detected by hand, even by the most experienced judges. The judges down-rated material from Treatment F—representing GLI only—to the *fair* category because it was considered to have a darker color than desired for salt-stock purposes. However, no marked color differences were obtained when salt-stock from all GLI treatments was de-salted and manufactured into processed dill pickles and sweet pickles. Furthermore, these pickles received a quality rating of *good* for *overall acceptability* by a panel of 10 judges representing three different pickle companies.

Total acidity, pH, and optical density of the brine samples. The 6 treatments were brined essentially the same except for the possible effect of added GLI and/or cucumber flowers in certain lots. The total acidity and pH of the brine samples from the 1st to the 10th day are given in Table 3. The rate of brine acid development in all treatments was very consistent and typical of a 25° salometer fermentation. A very rapid rise

TABLE 2
Pectinolytic and cellulolytic enzyme activity of the brines 24 hours after brining and percentage inactivation of the enzymes by grape leaf inhibitor (GLI)

Treatment ¹	Pectinolytic enzyme		Cellulolytic enzyme	
	Activity units/ml. ²	Inactivation by GLI %	Activity units/ml. ²	Inactivation by GLI %
A: Control, no flowers, no GLI.....	13	0	22	0
B: Control, flowers, no GLI.....	35	0	240	0
C: Flowers + 0.25 lb. GLI.....	20	43	172	28
D: Flowers + 0.75 lb. GLI.....	10	71	118	51
E: Flowers + 1.25 lbs. GLI.....	6	83	68	74
F: Control, no flowers + 1.25 lbs. GLI.....	2	85	5	77

¹ 1.25 lbs. cucumber flowers added per bbl. as indicated; GLI = grape leaf inhibitor added as indicated.

² Average of duplicate lots.

NB: The average firmness (in pounds) of the cured salt-stock from the various treatments was as follows: A, 19 lbs.; B, 13; C, 14; D, 17; E, 19; and F, 18.

in acid development occurred within 3 days of fermentation and maximum acidities from 0.51 to 0.62% were reached after 10 days. The increase in brine acidity was accompanied by a corresponding decrease in brine pH during the 10-day period. The pH values dropped from above 5 on the 1st day to 3.6 for all treatments on the 3rd day. The optical density (O.D. × 10) of the brine samples reached a peak between the 3rd to 5th day with values in the range of 3.0 to 4.0; this indicated an early, vigorous lactic acid fermentation.

Based on the above results, the addition of grape leaves appeared to exert no noticeable inhibiting effect on the lactic acid bacteria responsible for the acid fermentation in the various experimental treatments.

Commercial pectinase experiment. The pectinolytic enzyme

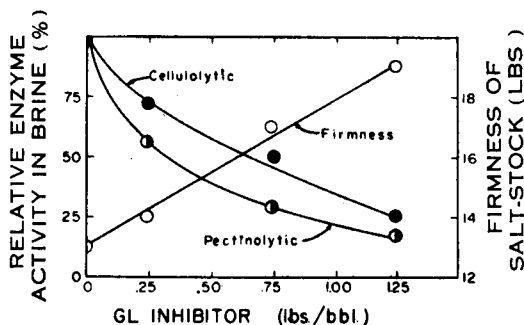


Figure 2. Effect of grape leaf inhibitor on maximum pectinolytic and cellulolytic enzyme activity in cucumber brines as related to firmness of cured salt-stock.

activity of the commercial material was extremely high and the addition of 8 grams per barrel was far in excess of that found in commercial brines with soft salt-stock. The use of 8 g. of enzyme without inhibitor (Treatment O) resulted in a maximum pectinolytic enzyme activity of 57,000 units at 24 hours; 100 units in the brine is usually sufficient to reduce salt-stock firmness 50%. The addition of 0.25 pound of GLI per barrel (Treatment P) reduced this activity 25% and 0.75 pound of GLI per barrel (Treatment Q) reduced the activity about 50%. With the exception of control Treatment N (without pectinase and without GLI), the salt-stock from the pectinase treatments was extremely soft.

Dried GLI experiment. Laboratory tests on the dried preparation made from grape leaves demonstrated that it was only about one-third as effective as an equivalent amount of frozen and thawed material in inhibiting pectinolytic enzyme activity. A limited brining study—using one level of dried GLI (equivalent to 1.25 lbs. of fresh leaves)—further revealed an even more drastic loss in ability to inhibit cellulolytic activity. In addition, salt-stock cucumbers from the above test showed no improvement in firmness over material from flower-added controls.

DISCUSSION

The inhibition of pectinolytic and cellulolytic activity by the crude brine extract of Scuppernong grape leaves offers a most promising lead to the ultimate chemical control of softening activity in commercial cucumber brines. It is obvious that the addition of such large quantities of grape leaves to commercial vats of cucumbers would not be practical. However, with further characterization and isolation of the active inhibitor substance, coupled with further investigations in the screening of plant materials to obtain higher concentrations of the active ingredient, this pectinolytic and cellulolytic inhibitor would be most valuable to the pickle industry and possibly other industries where the control of the degradation of pectin and cellulose is desirable.

The potential use of such an inhibitor might well extend into the fields of fungicides and insecticides. The relative freedom from disease and insect attack enjoyed by leaves of the Scuppernong (5) may well be the result of this naturally occurring enzyme inhibitor. Such a compound could be envisioned as inhibiting certain enzyme systems (i.e. cellulase) of the attacking fungi and insects and thereby limiting utilization of this plant material as a source of nutrients.

Information bearing on the purification, characterization, and chemical properties of the inhibitor(s) in grape leaves will be reported in another paper.

SUMMARY

Pectinolytic and cellulolytic enzymes in cucumber flowers, when added to small-scale fermentations, were effectively reduced in activity by the use of a crude extract of Scuppernong grape leaves (*Vitis rotundifolia*). There was no apparent influence of the grape leaf inhibitor (GLI) on the character of the acid fermentation as measured by total acidity, pH, and optical density of the brine. The reduction in activities of the two enzyme systems was directly related to the inhibitor concentration used; higher levels of inhibitor resulted in an increase in firmness of the fermented cucumbers (salt-stock). In general the judges at the pickle plant rated the salt-stock *good to excellent* as

TABLE 3
Total acidity and pH of the brines from the experimental treatments during 10 days of fermentation

Treatment ¹	Fermentation in days					Fermentation in days				
	1	2	3	5	10	1	2	3	5	10
	Total acidity as lactic ²					Brine pH ²				
	%	%	%	%	%					
A: Control, no flowers, no GLI.....	.08	.32	.37	.39	.54	6.2	3.7	3.6	3.6	3.5
B: Control, flowers, no GLI.....	.09	.43	.51	.51	.45	5.8	3.7	3.6	3.6	3.5
C: Flowers + 0.25 lb. GLI.....	.08	.28	.50	.52	.55	5.6	4.0	3.6	3.6	3.5
D: Flowers + 0.75 lb. GLI.....	.09	.27	.51	.56	.62	5.6	4.5	3.6	3.5	3.4
E: Flowers + 1.25 lbs. GLI.....	.08	.25	.54	.57	.55	5.4	4.4	3.6	3.5	3.4
F: Control, no flowers + 1.25 lbs. GLI.....	.06	.19	.50	.50	.48	5.9	4.7	3.6	3.6	3.5

¹1.25 lbs. cucumber flowers added per bbl. as indicated; GLI = grape leaf inhibitor added as indicated.
²Average of the duplicate lots.

to *acceptability for commercial use* and the only comment was "darker color" for material from some of the GLI treatments. However, when salt-stock was finished into processed dill pickles and sweet pickle products, there was no appreciable difference in color of the GLI treated stock over the control. Furthermore, the pickles made from all GLI lots received a quality rating of *good for overall acceptability*.

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