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Inhibition of Pectinolytic and Cellulolytic Enzymes in Cucumber Fermentations by Sericea

SUMMARY

Pectinolytic and cellulolytic enzymes in cucumber flowers, when added to small-scale cucumber fermentations, were effectively reduced in activity by the use of a brine extract of sericea (*Lespedeza cuneata* Don) and by a freeze-dried substance isolated from this plant. Reduction of enzyme activity in the fermenting brines was directly related to the inhibitor concentration used. Higher levels of the inhibitor resulted in an increase in firmness of the cured salt-stock cucumbers. The judges rated the salt-stock from all inhibitor treatments *good* to *excellent* as to acceptability for commercial use. The addition of sericea, either as the brine extract or the isolated substance, appeared to exert no lasting inhibitory effect on the lactic acid bacteria responsible for acid fermentation in the experimental brines.

INTRODUCTION

The softening of cucumbers brined under commercial conditions was first shown by Bell *et al.* (1950) to be enzymatic in nature and the direct result of hydrolytic action by an enzyme system similar in behavior to polygalacturonase. Later, it was demonstrated that cellulolytic enzyme systems are also present in curing brines and may contribute to the total softening action (Bell *et al.*, 1955; Etchells *et al.*, 1955a). Continued studies on this perplexing and important problem implicated filamentous fungi as the actual cause of softening spoilage. Moreover, the hydrolytic enzymes pectinase and cellulase, of fungal origin, were shown to be introduced into the curing brines chiefly by way of fungus-laden flowers that remain attached to the green cucumbers, and to a lesser extent by the cucumbers themselves (Etchells *et al.*, 1958a; Raymond *et al.*, 1959). This work also demonstrated that the maximum concentration of softening enzymes diffused out of the flowers and into the brine within 24–48 hr after the vats were filled. This finding permitted development of a simple but effective softening-spoilage control measure consisting of draining off the original enzyme-laden cover brine 36–48 hr after filling, and replacing it with a new brine (Etchells *et al.*, 1955a,b). However, it has been our belief that a more satisfactory approach toward eliminating or significantly reducing the concentration of softening enzymes in commercial cucumber brines would be to use specific, nontoxic inhibitors of plant origin; recent investigations in our laboratory have been directed toward this end.

Bell and associates (Bell and Etchells, 1958; Bell *et al.*, 1960) first demonstrated the presence of a naturally occurring pectinase and cellulase inhibitor in leaves of the grape. This was followed by a more extensive study where in the water-soluble extracts of a wide variety of plants were screened for their ability to inhibit these two enzyme systems (Bell *et al.*, 1962). Of the 61 plant species in 32 families examined, 8 species were found to be good sources of the pectinase inhibitor: grape, persimmon, dogwood, blueberry, sericea, blackberry, raspberry and rose. The first five species also gave strong inhibition of cellulase activity. A recent paper (Bell *et al.*, 1964) reported that the leaves of muscadine grape, persimmon, and the leaves and stems of sericea also provided good yields of a purified inhibitor substance. Preparations from these three plants, isolated by the caffeine-complex method (Barnes, 1956; Porter and Schwartz, 1962), were rated about equal in their inhibitory properties against the pectinase and cellulase enzyme systems.

The present investigation was undertaken to test the effectiveness of sericea prepara-

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tions (brine extracts and the isolated substance) in small-scale cucumber fermentations for the inhibition of added softening enzymes, pectinase, and cellulase. It was also of interest to correlate, if possible, any reduction of softening activity with improved firmness of the cured salt-stock cucumbers. A further objective was to determine if the sericea inhibitor exerted any influence on the character of the lactic acid fermentation.

MATERIALS AND METHODS

The inhibitor source for the softening enzymes was the forage crop sericea. This material, harvested from four fields near Raleigh and from two in the vicinity of Laurinburg, North Carolina, was first refrigerated for several hours at 4°C and then chopped into 4-to-6-inch lengths, packed in freezer bags, and held at −10°C. Later, the frozen sericea was shipped by air with dry-ice to the pickle plant just prior to preparation and use as a brine extract in the cucumber brining studies and referred to herein as FSI. Frozen sericea from the same sources was used to prepare the freeze-dried inhibitor substance later used in the brining tests and referred to herein as SI. Methods for harvesting, handling, storage, and preparation of the inhibitor substance from sericea have recently been described in detail by Bell *et al.* (1964).

The effect of this plant material on small-scale cucumber fermentations in 45-to-50-gallon-capacity barrels or fiber drums was followed as to: 1) pectinolytic and cellulolytic enzyme activity of the brines; 2) brine acidity and pH; 3) optical density of the brines; and 4) firmness and general quality of the brine-stock cucumbers. The procedures for making the above-named series of tests were given in an earlier study on the use of grape leaves in cucumber fermentations to inhibit softening enzymes (Etchells *et al.*, 1958b). That work also gave information directly applicable to the present investigation on: source, variety, and size of cucumbers used; collection and handling of cucumber flowers; preparation of the brine extract from plant material; and brining and sampling procedures.

The two commercial pectinases, 41 and 41P-conc, were kindly supplied by Rohm and Haas Company, Philadelphia, Pennsylvania. The experimental work was done during the 1961 and 1962 brining seasons at a commercial pickling plant located in northeastern Texas. Brine temperatures during the active fermentation period were in the range of 28–32°C.

Table 1 gives details concerning the treatments employed during the study, together with information on the basic brining procedure used for the small-scale fermentations.

RESULTS AND DISCUSSION

Pectinolytic and cellulolytic activities of the brine samples presented in Table 2 were usually found to be at their maximum level 24 or 48 hr after the cucumbers were covered with brine. For this reason, all enzyme inhibition percentages were based on measurements made at these sampling intervals. Increasing levels of sericea inhibitor (FSI) caused decreasing activity for both enzyme systems originating from 2 lb of added cucumber flowers. The highest level of FSI used (2.0 lb) reduced the pectinolytic and cellulolytic activities 94 and 70%, respectively, and the salt-stock firmness was within 1 lb of the control. The two lowers levels of FSI (0.5 and 1.0 lb) did not improve salt-stock firmness over that of the softening control (treatment A).

The three levels of commercial pectinase-41 added to the curing brines resulted in pectinolytic enzyme activity of a very high order (535 to $>$ 10,000 units/ml). For each case, this activity was completely inactivated by the addition of sericea inhibitor. Cellulolytic activity, although present in less than 100 units/ml, was likewise reduced 100%. The salt-stock firmness readings for all treatments in this portion of the 1961 season's study were essentially the same. The three pectinase-added lots gave values between 14 and 15 lb firmness; the same was true for those receiving the sericea inhibitor in addition to pectinase.

The experimental work for the 1962 season followed the same general plan as for the previous year except that the substance isolated from sericea (SI) was used for enzyme inhibition, and the softening enzyme load was increased by the addition of an extra pound of cucumber flowers. Pectinase-41 was replaced with a more concentrated preparation.

The pattern of inhibition of the two enzyme systems from cucumber flowers by the sericea inhibitor (SI) shown in Table 3 was essentially the same as that described for the 1961 season (Table 2) where the brine extract of sericea (FSI) was employed. For example, pectinolytic enzyme inhibition for the highest levels added of both forms of the

inhibitor (Table 3, treatment *F*, and Table 2, treatment *E*) was within the narrow range 87–94%; for cellulolytic activity, both sericea preparations caused 70% inhibition. The same relationship was also true for within the 1962 season (Table 3); here the

Table 1. Treatments employed in the small-scale cucumber fermentations with added softening enzymes (cucumber flowers or commercial pectinases) and sericea inhibitor [brine extract of fresh-frozen material (FSI) or isolated substance (SI)].

Treatment [a]	Softening enzyme added per barrel as:		Sericea inhibitor added per barrel as:	
	Cucumber flowers (lb)	Pectinase-41 (ppm)	Brine extract (FSI)[b] (lb)	Isolated substance (SI) (ppm)
1961 season, Model var. cucumber				
A	0.0		0.0	
B	2.0		0.0	
C	2.0		0.5	
D	2.0		1.0	
E	2.0		2.0	
F	0.0		2.0	
G		25	2.0	
H		25	0.0	
J		5	2.0	
K		5	0.0	
L		1	2.0	
M		1	0.0	
N[c]	2.0		2.0	
1962 season, Model var. cucumber		Pectinase-41-P conc. (ppm)		
A	0.0			0
B	3.0			0
C	3.0			5
D	3.0			25
E	3.0			50
F	3.0			100
G	3.0		2.0	
H		10		100
J		10		0
MR-17 var. cucumber				
AA	0.0			0
BB	3.0			0
KM		10		0
L		10		100

[a] Each treatment consisted of 200–225-lb lots of No. 1B size Model or MR-17 variety pickling cucumbers brined in 45–50-gallon barrels or polyethylene-lined fiber drums of similar capacity. The basic brining treatment consisted of a 25° salometer (6.6% salt) cover brine plus sufficient salt added on the false head to maintain that concentration at equalization (24–36 hr). The initial brine strength was then raised 5° sal (1.3%) per week to a holding strength of 60° sal (15.8%). This procedure represented commercial practice at the plant where the work was done. In 1961, all treatments were in duplicate; in 1962, treatments A, B, H and J were duplicated.

[b] For all FSI-added lots except "N," the amounts indicated of fresh-frozen sericea were first blended in 1-gallon of 25° sal brine for 2 min, then the insoluble material was filtered out through cheesecloth and the brine extract was added to the cucumbers as part of the cover brine.

[c] For this lot, the sericea was not blended in brine to produce the extract but merely soaked 2 hr in 1 gallon of 25° sal brine and the total content (brine + sericea) added as part of the cover brine.

Table 2. Pectinolytic and cellulolytic enzyme activity of brines 24 hours after brining, and firmness of the cured, salt-stock pickles—1961 season.

	Treatment[a]	Pectinolytic enzyme		Cellulolytic-enzyme		Firmness of cured salt-stock (lb)
		Activity (unit/ml)	Inhibition by sericea (%)	Activity (unit/ml)	Inhibition by sericea (%)	
A	Control, no flowers, no FSI	2	0	16	0	16
B	Flowers, no FSI	98	0	333	0	13
C	Flowers + 0.5 lb FSI	70	29	308	8	13
D	Flowers + 1.0 lb FSI	54	45	292	12	13
E	Flowers + 2.0 lb FSI	6	94	99	70	15
F	No flowers + 2.0 lb FSI	0	0	1	0	16
G	Pectinase, 25 ppm + 2.0 lb FSI	1	100	4	95	15
H	Pectinase, 25 ppm, no FSI	> 10,000	0	73	0	14
J	Pectinase, 5 ppm + 2.0 lb FSI	0	100	5	89	15
K	Pectinase, 5 ppm, no FSI	2,500	0	44	0	14
L	Pectinase, 1 ppm + 2.0 lb FSI	0	100	6	84	15
M	Pectinase, 1 ppm, no FSI	535	0	38	0	15
N	Flowers + 2.0 lb FSI	51	48	305	8	15

[a] Cucumber variety MR-17 used in all 1961 brining tests.

inhibitory action for SI and FSI (treatments *F* and *G*) was considered to be the same. Cucumber flowers alone (treatment *A*) reduced the salt-stock pickle firmness from 18 to 12 lb (33% loss). Five ppm of SI did not inhibit softening enzyme activity, and this was reflected by a 6-lb loss in firmness of the pickles, which was equivalent to that shown for the flower-added control. However, increasing levels of the inhibitor (25, 50, and 100 ppm) added to brines resulted in higher firmness values (14–16 lb) for the cured salt-stock pickles.

In considering the treatments with 10 ppm pectinase, this enzyme preparation was completely inactivated by 100 ppm SI, and the salt-stock pickles were adequately protected from softening action. This was true for fermentations of two cucumber varieties, Model and MR-17. In the absence of the inhibitor, the loss in firmness for both varieties amounted to 6 lb (33%). Although

Table 3. Pectinolytic and cellulolytic enzyme activity of brines 48 hours after brining, and firmness of the cured, salt-stock pickles—1962 season.

	Treatment	Pectinolytic enzyme		Cellulolytic-enzyme		Firmness of cured salt-stock (lb)
		Activity (unit/ml)	Inhibition by sericea (%)	Activity (unit/ml)	Inhibition by sericea (%)	
Model var. cucumber:						
A	Control, no flowers, no SI	15	0	12	0	18
B	Control, flowers, no SI	93	0	300	0	12
C	Flowers + 5 ppm SI	90	3	255	15	12
D	Flowers + 25 ppm SI	80	24	260	14	14
E	Flowers + 50 ppm SI	34	64	215	28	14
F	Flowers + 100 ppm SI	12	87	90	70	16
G	Flowers + 2.0 lb FSI	10	90	86	71	17
H	Pectinase, 10 ppm + 100 ppm SI	1	100	1	98	18
J	Pectinase, 10 ppm, no SI	> 10,000	0	51	0	12
MR-17 var. cucumber:						
AA	Control, no flowers, no SI	64	0	29	0	17
BB	Control, flowers, no SI	116	0	250	0	14
KM	Pectinase, 10 ppm, no SI	> 10,000	0	60	0	11
L	Pectinase, 10 ppm + 100 ppm SI	2	100	1	98	16

both varieties responded in the same manner as to the degree of softening resulting from 10 ppm of the commercial pectinase, MR-17 var. may have offered some resistance to softening by the pectinolytic activity of cucumber flowers. Model var. was reduced in firmness by flowers by 6 lb (treatment *B*) more than the control (treatment *A*), but the reduction in firmness for MR-17 var. (treatment *BB*) as compared to its control (treatment *AA*) was only one-half that amount even though the enzyme activity of the brine was somewhat higher (116 units vs. 93). It will be recalled that MR-17 var. was used throughout in the 1961 brining studies, and that the reduction in brine stock firmness attributed to cucumber flowers (Table 2, treatment *B*) was the same (3 lb) as obtained with this variety for the 1962 season.

Fig. 1 shows the influence of both forms of sericea inhibitor (the brine extract and the isolated substance) on relative pectinolytic and cellulolytic enzyme activities at their maximum level in the flower-added brines. As the inhibitor (FSI or SI) concentration was increased, the activity of both enzyme systems decreased. Further, within a given enzyme system, both forms of the inhibitor produced similar inhibition curves. However, the cellulolytic enzyme system appeared to be less sensitive to the inhibitory properties of sericea than the pectinolytic.

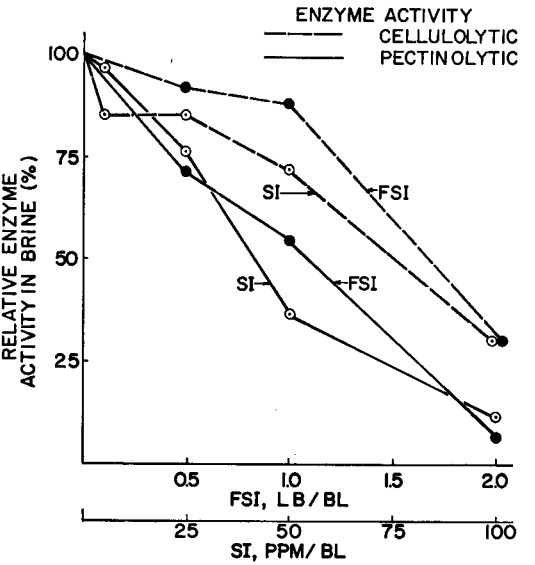

Fig. 1. Effect of sericea inhibitor on maximum pectinolytic and cellulolytic enzyme activity in cucumber brines. FSI = crude, brine-extract inhibitor solution prepared from fresh-frozen sericea and introduced in the cucumber cover brine in equivalent extracted amounts (lb/bl) of fresh material as indicated; SI = isolated inhibitor substance prepared from fresh-frozen sericea and introduced in the cucumber cover brine (ppm/bl) as indicated.

Table 4 shows total acidity and pH of brine samples from treatments typical of those examined during the two-season study. The values represent tests made on samples collected during the first 10 days after the

Table 4. The acidity and pH of brines from experimental treatments during the 10 days of fermentation.

Treatment	Fermentation in days: Total acidity as lactic 1 (%)	2 (%)	3 (%)	5-6* (%)	10 (%)	Fermentation in days: Brine pH 1	2	3	5-6*	10
1961 season:										
A Control, no flowers, no inhibitor	.08	.29	.43	.70	.77	5.45	4.12	3.87	3.50	3.50
B Control, flowers, no inhibitor	.07	.27	.52	.70	.80	5.77	4.47	3.72	3.50	3.50
C Flowers + 0.5 lb FSI	.07	.13	.37	.62	.75	5.85	5.70	3.90	3.50	3.50
D Flowers + 1.0 lb FSI	.08	.12	.22	.60	.66	5.90	5.65	4.15	3.50	3.55
E Flowers + 2.0 lb FSI	.08	.14	.31	.58	.72	5.90	5.30	3.90	3.50	3.55
1962 season:										
A Control, no flowers, no inhibitor	.02	.17	.31	.41	.39	6.25	4.47	3.73	3.57	3.45
B Control, flowers, no inhibitor	.02	.12	.38	.43	.55	6.75	4.80	3.80	3.75	3.40
C Flowers + 5 ppm SI	.02	.10	.35	.41	.52	6.75	4.90	3.85	3.75	3.40
D Flowers + 25 ppm SI	.02	.12	.36	.50	.46	6.80	4.75	3.90	3.65	3.50
E Flowers + 50 ppm SI	.03	.05	.19	.50	.56	5.70	5.90	4.35	3.65	3.40
F Flowers + 100 ppm SI	.02	.03	.17	.48	.48	6.10	5.90	4.50	3.65	3.55

*Brine samples taken on fifth day in 1962; sixth day in 1961.

cucumbers were brined; this time interval adequately covers the active acid fermentation period under the brining conditions employed. Within each season, the maximum amount of brine acid developed by the lactic fermentation was comparable for all treatments. The higher inhibitor levels (1961, *D* and *E*; 1962, *E* and *F*) appeared to retard the onset of acid production, but this initial delay was rapidly overcome and a vigorous lactic fermentation ensued. The resultant final acidities were essentially the same as in the control treatments, and typical of natural fermentations at 25° salometer. In each treatment, an increase in brine acidity was accompanied by a corresponding decrease in brine pH during the active fermentation period. By the tenth day, the pH values for all treatments had dropped to minimum levels in the range of 3.40–3.55.

The optical density (OD × 10 at 650 mμ) of the brine samples reached peaks for all treatments between the third and fifth days of fermentation. In general, the values obtained for the inhibitor-added treatments were lower (2–4 range) than for the controls (3–7 range). This finding might reflect a decrease in certain groups composing the total microbial population present in brines receiving the sericea inhibitor. However, final brine acidities and pH's would support the view that the addition of sericea, either as the brine extract (FSI) or the isolated substance (SI), appeared to exert no deleterious or lasting inhibiting effect on the lactic acid bacteria responsible for the acid fermentation in the experimental treatments.

A panel of experienced salt-stock judges, representing several Texas pickling companies, evaluated coded 50-lb lots of the material from the 1961 and 1962 experimental treatments listed in Tables 2 and 3. In brief, all inhibitor-treated lots were rated *good* to *excellent* as to acceptability for commercial use. This was the same rating given salt-stock from the control treatments.

Additional brining experiments with the isolated sericea substance (SI) were conducted during the 1962 season at two cooperating plants in northern production areas, Ohio and Minnesota. In all, twenty-six 50-gallon lots and four small commercial tanks were employed, together with inhibitor levels of zero, 5, 10, 25, 50, and 100 pm. A detailed account of this work will be given in a separate report; however, it is pertinent that the cured brine-stock from all inhibitor-added lots was again rated *good* to *excellent* as to acceptability for commercial use.

The two commercial pectinases used in the current brining tests gave extremely high values for pectinolytic enzyme activity, ranging from 535 to > 10,000 softening units per milliliter of brine. One might have expected such enzyme concentrations in the curing brines to have completely softened the brined cucumbers, particularly when our experience has shown that 150–200 softening units from cucumber flowers are usually sufficient to reduce firmness 50% or more. Earlier research on the behavior of pectinase in the presence of salt (Bell and Etchells, 1961) established that, as the salt content of cucumbers was increased, the softening action of three pectinases (AP-46, polygalacturonase, and filtrates from fungi) decreased according to a first-order reaction. This important relationship of the action of salt on softening also applies to the enzyme systems found in cucumber flowers; thus it does not provide an explanation for the discrepancy of the degree of softening action between flowers and pectinases experienced in the present study. Rather, a clearer understanding of the chemical composition and properties of pectinases is essential to determining the optimum conditions under which such complex fungal mixtures will readily soften plant tissue, particularly brined cucumbers undergoing fermentation. Recently, McClendon and Hess (1963) studied eight commercial pectinases with pH-gradient chromatography and found that all were different as to enzymic composition.

Although Etchells *et al.* (1958b) showed that grape leaves effectively inhibit pectinolytic and cellulolytic enzymes and thus protect brined cucumbers from their softening action in small-scale fermentations, their limited supply would exclude them as a source in quantity for the inhibitor substance. The same would be true for persimmon leaves, which were mentioned by Bell *et al.* (1964) as a good source of the inhibitor substance. The forage crop sericea, however, is another matter. This perennial is widely

grown as a forage crop for hay and pasture and lends itself to large-scale production and harvesting. In the southeastern states, sericea is grown on an estimated 1 million acres. Furthermore, it is grown on land of low fertility and low organic matter, and few plants can compete with it in yield per acre and drouth resistance (Cope, 1964). Thus it is believed that, based on present results demonstrating the effectiveness of sericea for inhibition of softening enzymes, plus its availability as source in quantity, this plant offers high potential for ultimately eliminating softening spoilage of commercially brined cucumbers.

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