PECTINASE AND CELLULASE ENZYME INHIBITOR FROM SERICEA AND CERTAIN OTHER PLANTS[1]

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ABSTRACT

A water-soluble substance isolated from the leaves of seven plant species is shown to inhibit the fungal hydrolytic enzymes, pectinase and cellulase. Three plants—sericea, muscadine grape, and persimmon—were found to be good sources of the inhibitor. Preparations of all three, isolated by the caffeine-complex method, were rated about equal in enzyme inhibition, and their chemical and physical characteristics were found to be essentially the same. When sericea was harvested in the field with a silage cutter, inhibitor activity was rapidly lost as compared to harvesting by cutting the whole stalk. Total tannin content did not appreciably change under any of the conditions of harvesting or dehydration. Grape-leaf and sericea-enzyme inhibitor are both of high molecular weight (14,000–20,000) and have certain chemical reactions that place them as condensed polymers of catechins or leucoanthocyanidins.

Introduction

The water-soluble leaf extracts from 61 plant species in 32 families were screened by BELL *et al.* (1962) for their ability to inhibit two hydrolytic fungal enzymes, cellulase and pectinase. The leaves from eight out of 29 species which indicated inhibition were considered good sources of pectinase inhibitor. These were the leaves of muscadine grape, persimmon, dogwood, blueberry, sericea, blackberry, raspberry, and rose. The first five of the eight plant species were listed as also giving strong inhibition to cellulase. Earlier studies by BELL and associates (BELL and ETCHELLS, 1958; ETCHELLS *et al.*, 1958; BELL, AURAND, and ETCHELLS, 1960) have reviewed the problem of softening of brined cucumbers by pectinolytic and cellulolytic enzymes and the economic importance in finding a suitable enzyme inhibitor to control this spoilage problem. These enzymes were found to be introduced into the fermentation by the mold-laden flowers attached to the green cucumbers. Water-soluble extracts of muscadine grape leaves (*Vitis rotundifolia* Michx.) were reported by ETCHELLS, BELL, and WILLIAMS (1958) to prevent enzymatic softening of cucumbers, but this procedure was not considered to be commercially practical. The chemical identity of the active component from grape leaves has not been established (BELL and ETCHELLS, 1958; BELL *et al.*, 1960; PORTER *et al.*, 1961; PORTER and SCHWARTZ, 1962).

The inhibitor substance from muscadine grape leaves was isolated by PORTER and SCHWARTZ (1962)

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by complexing the material with caffeine, extracting in chloroform, and freeze-drying the water-soluble material. They described the material as a "condensed tannin." Some tannins and other phenolic compounds have been reported by others (COLE, 1958; HATHWAY and SEAKINS, 1958; POLLARD, KIESER, and SISSONS, 1958) as pectinase inhibitors.

Inhibition of cellulases by physical factors, chemical action, and natural-occurring inhibitors from plants was thoroughly reviewed in 1963 by MANDELS and REESE. Two important relationships concerning cellulase inhibition were pointed out by these reviewers; one was the diverse array of chemicals that can be added to the fungal cellulases in fairly high concentrations without causing inactivation, and the second was that cellulases from different organisms differed greatly in their resistance to various inhibitors. The latter observation had been reported earlier (BELL *et al.*, 1960) in connection with studies on grape-leaf inhibitor. Commercial cellulase preparations were not appreciably inhibited by grape-leaf extracts, whereas certain fungal filtrates and cucumber-flower cellulase were inactivated completely.

The present study was initiated to isolate the pectinase and cellulase inhibitor from seven species of plants. Crude water extracts of six of these species were designated earlier by BELL *et al.* (1962) to be good sources of the inhibitor. Special attention was given to one species, sericea (*Lespedeza cuneata* Don.), because of its potential as a source of good quantities of the inhibitor to be used in controlling softening-type spoilage in cucumber fermentations. Sericea is a perennial lespedeza used as a forage crop for hay and pasture and is grown on land of low fertility and organic matter in the southeastern part of the United States. The high tannin content of this forage has been related to its low palatability and feeding value (STITT and CLARKE, 1941).

Material and methods

PLANT MATERIALS.—Mature leaf samples, about 500 g each, were collected, with one exception, from the greenhouse and the Method Horticultural Station, North Carolina Agricultural Experiment Station, and classification of the plant species followed BAILEY (1949). The fresh sericea samples were collected in September at the Baker farms of McNair Seed Company, Laurinburg, North Carolina. The experiments designed to test the influence of harvesting methods and the effect of dehydration on sericea inhibitors were done at the commercial dehydration plant of this company. For all species except sericea only leaves were used for inhibitor extraction. For sericea, stems with leaves were cut either with a hand sickle about 8 inches above the ground or by a mechanical silage cutter. All plant samples were taken to the laboratory in 4 hr or less after collection, and care was taken to prevent them from heating. The samples were washed in tap water, air-dried to about the original fresh weight, and stored in polyethylene bags at −10° C.

ISOLATION OF INHIBITOR.—The method of BARNES (1956), as described by PORTER and SCHWARTZ (1962), was followed with but slight modification. Aqueous extracts of each frozen plant were prepared by blending 40 g of shredded leaves in 300 ml distilled water for 3 min. For larger extractions, 250 g of the plant were macerated in 2 liters of water using the 1-gal capacity Waring blendor. The slurry was then pressed through several thicknesses of cheesecloth and the liquid centrifuged for 15 min at 3000 rpm (2250 × g). For larger extractions, the Sharples Super-Centrifuge, Model T-1 (Sharples Corporation, Philadelphia, Pennsylvania) was used to remove the suspended plant particles. The clarified extract was cooled to 15° C and recentrifuged. The clear aqueous extract was cooled to 5° C and caffeine solution (15 g/liter) at 12° C was added in amounts of 200 ml per 300 ml of plant extract. The mixing solutions were stirred constantly while the caffeine solution was added, and the temperature was maintained between 7° and 10° C. The caffeine-inhibitor complex was precipitated immediately and settled rapidly in the container. About two-thirds of the supernatant liquor was decanted, and the remainder was separated by centrifugation. The caffeine-inhibitor complex was resuspended in distilled water (volume used was one-tenth that of the original plant extract complexed with caffeine) by blending, and the water suspension was transferred to liquid-liquid extractor which contained sufficient chloroform to float the water suspension. The chloroform was allowed to percolate through the water-suspended complex for 8 hr for a small extraction and 20 hr for a larger one, thus removing the caffeine and causing the inhibitor substance to become water-soluble. Next, the water layer was removed, filtered through Whatman no. 5 paper, and placed in a vacuum flash-evaporator and the total volume reduced by approximately one-half (water-bath temperature about 50° C). The inhibitor solution was divided in about 100-ml amounts, each placed in 2-liter, round-bottom, ground-joint flasks and subjected to freeze-drying in the usual manner. The resulting dry inhibitor substance was light gray to tan in color and extremely fluffy and light. It was stored in a tightly sealed brown bottle in a cool place.

Water extracts of sericea (10 g/100 ml) were used in some tests, and the extraction method previously described (BELL *et al.*, 1962) was used.

ENZYME SOURCES.—Crude enzyme solutions of pectinase and cellulase were prepared from partially dried cucumber flowers (CF) as previously described

(BELL and ETCHELLS, 1958; BELL *et al.*, 1960). Pectinase 46AP no. 32 was also used; this was supplied by Rohm and Haas Company, Philadelphia, Pennsylvania.

MEASURING ENZYME ACTIVITY AND INHIBITION.— Pectinase, which hydrolyzes the glycosidic bonds of pectic acid, and cellulase, which hydrolyzes the glucosidic bonds of a soluble cellulose derivative (sodium carboxymethylcellulose) were determined by viscosity methods reported in earlier papers (BELL and ETCHELLS, 1958; BELL *et al.*, 1960; BELL *et al.*, 1962). A value of 100 units was established to equal 50% loss in viscosity in 20 hr of an enzyme-substrate mixture at 30° C and buffered at pH 5.0. The percentage loss in viscosity was converted to units by using a standard enzyme curve for each enzyme assayed. Plant extracts or the isolated inhibitor substances were tested for inhibition by mixing two parts of enzyme solution with one part of leaf extract; for controls, water was used in place of the extract and/or the enzyme solutions. In a previous report (BELL *et al.*, 1962), it was shown that increasing levels of grape-leaf inhibitor reduced both pectinase and cellulase enzyme activity according to a first-order-type reaction. Enzyme inhibition is expressed as follows: 0%–25% is considered doubtful to negative inhibition; 25%–60% = 1+ (weak); 60%–80% = 2+ (moderate); 80%–90% = 3+ (strong); and greater than 90% = 4+ (very strong).

LEUCOANTHOCYANIDINS AND CATECHINS TESTS.— The vanillin-HCl reaction as proposed by BATE-SMITH and LERNER (1954) was used with slight modification. About 10 mg of the freeze-dried inhibitor substance was dissolved in 6 ml methyl alcohol and four drops of concentrated HCl were then added. This solution was treated with 3–5 drops of saturated vanillin in ethyl alcohol. The development of a deep cherry-red color immediately following the addition of the vanillin indicates the presence of leucoanthocyanidins or catechins. A second test by BATE-SMITH (1954) was also used with modification. About 100 mg of the inhibitor substances in 6 ml 2.V HCl was boiled for 20 min in a water bath, then cooled, and 10 ml of isoamyl alcohol was added. The mixture was transferred to a separatory funnel and shaken. The isoamyl alcohol was drawn off, and the water phase was washed a second and third time with 15 ml of isoamyl alcohol. The isoamyl alcohol fractions containing the pigment were combined, and a deep cherry-red color in the alcohol indicated the presence of the above compounds. Absorption spectra were observed for the alcohol fraction using a Beckman Model DK-2 spectrophotometer.

TABLE 1

PECTINASE ENZYME INHIBITOR CONCENTRATION IN LEAVES OF SEVEN SPECIES OF PLANTS USING CAFFEINE-COMPLEX ISOLATION METHOD

PLANT		INHIBITOR SUBSTANCE (FREEZE-DRIED) PER 100 G FRESH WEIGHT OF PLANT (MG)	DEGREE OF INACTIVATION OF PECTINASE ENZYMES[a]	
Scientific name	Common name		46AP[b]	CF[b]
Lespedeza cuneata Don.	Sericea	1,380	4+	4+
Vitis rotundifolia Michx.	Muscadine grape	1,644	4+	4+
Diospyros virginiana L.	Persimmon	1,309	4+	4+
Cornus florida L.	Dogwood	150	4+	3+
Rubus strigosus Michx.	Red raspberry	54	3+	2+
Rubus occidentalis L.	Black raspberry	41	3+	2+
Rose odorata Sweet	Rose	41	4+	2+

[a] 4+ = greater than 90% inhibition; 3+ = 80%–90%; 2+ = 60%–80%; 1+ = 25%–60%. – = negative to doubtful or less than 25%.

[b] 46AP supplied by Rohm and Haas Company, Philadelphia; CF = cucumber-flower extract.

Results and discussion

ISOLATION AND CONCENTRATION OF ENZYME INHIBITOR.— In earlier studies the authors (BELL *et al.*, 1962) screened leaf extracts from 61 plant species in 32 families for pectinase and cellulase enzyme inhibition. They reported that the leaf extracts from 29 species exhibited some degree of pectinase inhibition and those from eight species gave sufficient activity (3+ and 4+ degree of inhibition) to warrant further investigation. The cellulase inhibition by the different species was less pronounced, and only five of the eight plants gave strong inhibition for the cellulase enzyme. For the present study, the selection of the seven species was based on their potential as good sources for both the pectinase and the cellulase inhibitor. The isolated enzyme-inhibitor concentration in milligrams per 100 g of plant material and the degree of pectinase inhibition on two pectinase enzyme sources are given in table 1. The amount of

inhibitor prepared ranged from 45 mg for both raspberry plants to over 2 g for persimmon, muscadine grape, and sericea. Samples from the last three gave not only the highest concentration but also the highest degree of pectinase inhibition. The red and black raspberry samples and rose samples gave very low yields (about 3%) as compared to sericea, muscadine grape, and persimmon; dogwood was about 10% of the latter three. It is apparent that three of the plants tested—sericea, muscadine grape, and persimmon—should provide good sources for isolating the enzyme inhibitor by the caffeine-precipitation method.

SERICEA ENZYME INHIBITOR.—Of the three plants with the highest yields in inhibitor concentration into two equal parts; one set was frozen immediately with dry ice, and the other was placed in paper bags (table 2). The results of the enzyme-inhibition tests demonstrated that a very rapid loss in inhibitor activity was caused by the silage cutter crushing and bruising the stems and leaves. If the silage-cut sericea was frozen in the field (sample 2[a], table 2), the loss in inhibitor activity was retarded; but when the cut sericea was held 75 min before freezing, the inhibitor activity was almost completely lost. Inhibitor activity of sericea was best retained by cutting the plant with a sickle about 6–8 inches from the ground, delivering it to the laboratory in 2–6 hr, then freezing it in plastic bags. Immediate freezing of sickle-cut sericea with dry ice did not increase the inhibitory

TABLE 2

ENZYME INHIBITOR CONCENTRATION OF SERICEA FORAGE AS INFLUENCED BY HARVESTING METHODS AND BY COMMERCIAL DEHYDRATION

SAMPLE NO.	SERICEA TREATMENT AND SOURCE, HARVESTING METHOD, AND COLLECTION OF SAMPLES	DEGREE OF CF ENZYME INACTIVATION[a]				TANNIN[c] (b) (%)
		Pectinase		Cellulase		
		(a)[b]	(b)[b]	(a)[b]	(b)[b]	
1	Field, stalk cut 6–8 inches from ground with sickle	4+	4+	4+	4+	8.81
2	Field, stalk cut 6–8 inches from ground with silage cutter, sampled after 5 min	2+	1+	1+		8.94
3	Same as 2, sampled from wagon after 60 min	1+	—	—		
4	Same as 2, sampled from wagon after 75 min	1+	1+	—		
5	Same as 2, sampled from wagon at dehydration plant after 105 min	1+	—	—		
6	Following dehydration, 115 min after harvest	—	—	—		8.63
7	Following dehydration and pelletizing	—	—	—		7.46

[a] See table 1, n. b, for key to enzyme inhibition.

[b] (a) = sericea samples frozen with dry ice in field at time of collecting; (b) duplicate sericea samples placed in paper bags and returned to laboratory at air temperature (about 85° F).

[c] Percentage on dry-weight basis; averages of four determinations. HCl-formaldehyde method used to determine tannin.

(table 1), sericea forage offered outstanding advantages for large-scale production. Hundreds of pounds of fresh plant material would be needed for inhibitor extractions, and it has been estimated (Dr. W. A. COPE, Department of Crop Science, North Carolina Agricultural Experiment Station, Raleigh) that the southern states have over 1,000,000 acres planted in this forage crop. North Carolina has large acreages where this plant is cut, dried, pelleted, and sold commercially as animal feed.

The influence of harvesting methods and dehydration of the sericea forage were investigated in a field experiment near Laurinburg, North Carolina. The study was carried out at a time when the crop was being mechanically harvested by a power-drawn silage cutter. The cut material was hauled to the commercial dehydration plant for drying. Seven sericea samples were collected during the harvesting operation, and each of these was divided immediately activity (table 2) over that frozen 2–6 hr after cutting. The commercially dehydrated sericea, both the powdered and pelletized samples, were found to be negative for enzyme inhibition. This was not surprising, because the freshly cut plant material going into the drying drums was also negative.

The pectinase enzyme inhibitor from grape leaves has been described as a "condensed tannin" (PORTER and SCHWARTZ, 1962); therefore, the total tannin content of the different sericea samples was investigated. It was found to range from 7.46 to 8.94% (dry-weight basis) with no apparent relationship between loss of enzyme-inhibitor activity and tannin content (table 2).

ACTIVITY AND CHARACTERISTICS OF ISOLATED ENZYME INHIBITOR.—All freeze-dried preparations of sericea were shown to be most active for pectinolytic and cellulolytic enzyme inhibition. A concentration of 100 ppm in final inhibitor-enzyme-substrate con-

sistently resulted in more than 95% inhibition of pectinase and cellulase enzyme (CF) systems. Commercial pectinase (46AP) was also very sensitive to inhibition by the freeze-dried material; 25 ppm gave more than 90% inhibition. Small increments of the isolated inhibitor gave marked reduction in both pectinase and cellulase activity, and a plot of the logarithm of the inhibitor concentration against percentage reduction of enzyme activity gave a straight line, thus suggesting a first-order reaction. This had been reported earlier for the pectinase and cellulase inhibitor from grape leaves (BELL *et al.*, 1962): 5 ppm of the inhibitor substance in the final enzyme-substrate solution reduced pectinase (CF) enzyme activity about 50%.

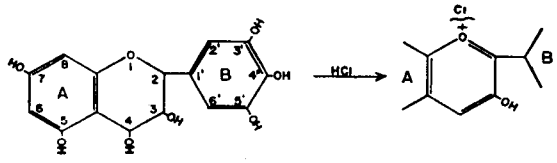

FIG. 1.—Basic structure of leucoanthocyanidins with conversion to pigmented form as anthocyanidins (ROBINSON, 1963). Leucodelphinidin is one of three types that differ in number of hydroxyl groups in B ring. Catechins are similar compounds which differ only in C-4 position with a hydrogen replacing OH group. Catechins are also colorless and form cherry-red color with HCl. Enzyme inhibitor is probably a polymer of above basic structure giving molecular weight of about 14,000.

The inhibitor substance isolated from sericea was soluble in water, methanol, and ethanol but insoluble in ethyl acetate, acetone, benzene, ether, chloroform, and glacial acetic acid. The preparation gave a blue color to methanolic ferric chloride solutions, as do many natural tannins. These chemical properties also were found for preparations made from grape and persimmon leaves.

The pectinase-inhibiting substance of grape leaves was non-dialyzable through a cellophane membrane against either running tap or distilled water (BELL and ETCHELLS, 1958). This indicated a minimum molecular weight on the order of 10,000. Sedimentation studies on the isolated pectinase inhibitor from the grape leaves, as described by PORTER and SCHWARTZ (1962), indicated that there is a broad range of molecular weights, and their light-scattering measurements gave an average molecular weight of 250,000 with a range from about 10,000 to figures in the millions. The molecular-weight determinations of our freeze-dried preparations of sericea and grape-leaf inhibitor were made by Mr. WILLIAM B. CARNEY, Seed Pioneering Research Laboratory, Southern Utilization Research and Development Division, New Orleans, Louisiana. In a personal communication he reported the following: Two per cent solutions of the inhibitors in distilled water were static when dialyzed against distilled water for 2 days. The two solutions were centrifuged for 10 min at 10,000 rpm to remove undissolved particles. The supernatants were then run on the Spinco Model E ultracentrifuge at 59,780 rpm, with the pictures being made at 8-min intervals, the first exposure coming 16 min after the machine had reached top speed. The calculated estimate of the sedimentation coefficient for the sericea inhibitor (B sample) is 2.1 Svedberg units, and for the grape inhibitor, 2.5 Svedberg units. Assuming certain factors about the shape of the molecule and the frictional ratio, the molecular weights would be about 14,000–16,000 for the sericea inhibitor and about 17,000–20,000 for the grape inhibitor.

CHEMICAL TESTS FOR LEUCOANTHOCYANIDINS AND CATECHINS.—These compounds are differentiated from other flavonoids and tannin-like substances in that they give a red-color reaction with vanillin and concentrated HCl (BATE-SMITH and LERNER, 1954). This reaction depends on 5–7 dihydroxy substitution of the A ring (fig. 1) and a carbonyl group at C-4 which interferes with the reaction; thus flavones and flavanones do not react (ROBINSON, 1963). Catechins differ from leucoanthocyanidins only in a single hydrogen in place of an aliphatic hydroxyl group in the C-4 position. The isolated inhibitor substances and certain phenolic compounds were treated with vanillin-HCl and observed for color reaction and maximum absorption in ethyl alcohol (table 3). Sericea, grape, persimmon, and catechin gave identical cherry-red color and maximum-absorption peaks, which indicated their closely related chemical structure. To confirm this, inhibitor preparations of sericea, grape, and persimmon were boiled with 2 *N* HCl and the pigments extracted in isoamyl alcohol. Each preparation showed a deep cherry-red color in the alcohol and two absorption peaks (455 and 558 μ) which were identical for the three preparations. No color developed in tests with catechol or tannic acid.

TABLE 3

RESULTS OF TEST FOR LEUCOANTHOCYANIDIN AND CATECHIN

Preparation	Color	Maximum absorption (mμ)
Sericea	Cherry red	495
Grape	Cherry red	500
Persimmon	Cherry red	500
Catechol	None	
Catechin	Cherry red	500
Tannic acid	None	

The basic structure of the enzyme inhibitor from

sericea, grape, and persimmon is identified as having 15 carbon atoms with a flavonoid skeleton. There is strong indication that at least part of the inhibitor molecule is a leucoanthocyanidin. Leucoanthocyanin (leucodelphinidin-3-glucoside) was recently (ITO and OSHIMA, 1962) identified in the methyl alcohol extraction from the unripe Japanese persimmon. Further, the high molecular weights (about 16,000) of the sericea and grape inhibitor substances would indicate a probable polymeric structure, and such polymers have recently been proposed for the catechins and leucoanthocyanidins. The C-1 and C-6 or C-2 and C-3, positions of two monomers, have been suggested as possible sites of condensation (ROBINSON, 1963), and it has been further suggested that polymers of this sort, involving unknown units of catechins or leucoanthocyanidins, constitute a general group of compounds known as "condensed tannins" or "phlobatannins." As added evidence that these compounds play a role in enzyme inhibition, COLE (1958) found that the production of pectinase activity by the brown-rot producing fungus, *Sclerotinia fructigena* Aderh. and Ruhl., in apples is inhibited by polymerized, oxidized leucoanthocyanins.

In a further attempt to purify the enxyme-inhibitor preparation of sericea, water solutions of the isolates were passed through a gel column of Sephadex G-25. The inhibitor material which passed through the column was almost completely white in color when freeze-dried and gave almost complete inhibition to pectinase and cellulase. The material darkened to a light-brown color on standing. The complete identification of the pectinase and cellulase inhibitor will require a number of chemical and physical methods using spectral analysis investigations with infrared, ultraviolet, and nuclear resonance to determine the presence or absence of certain structures.

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