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ENZYME INHIBITOR FOR PREVENTING  
SOFTENING IN BRINED FOODS

Thomas A. Bell, John L. Etchells, and William W. G. Smart, Jr., Raleigh, N.C., assignors to the United States of America as represented by the Secretary of Agriculture

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A non-exclusive, irrevocable, royalty-free license in the invention herein described, throughout the world for all purposes of the United States Government, with the power to grant sublicenses for such purposes, is hereby granted to the Government of the United States of America.

This invention has as its objective the use of an enzyme inhibiting material isolated and concentrated from the forage plant sericea (*Lepedeza cuneata*) to prevent "softening" in the brining of cucumbers and other vegetables and fruits, namely cherries, olives, onions, cauliflower, sweet and hot peppers, tomatoes, okra, green beans carrots, citron, and watermelon and cantaloupe rinds. It is known that the softening of brined cucumbers, this applies also to other vegetables and fruits being processed under commercial conditions, is caused by activity within the fleshy structure of the cucumber of certain hydrolytic enzymes such as, for example, the pectinolytic and cellulolytic enzymes. Softening occurs when the pectic substance, which functions somewhat as a cementing agent for the cells of plant tissue, is broken down. Thus, softening can be viewed as being intimately connected with a group of hydrolytic enzymes.

Pectic substances are essentially long chain polymers of D-galacturonic acid, and are connected by 1,4-glycosidic bonds. Pectin differs from pectic acid in that about 3 of every 4 residues are esterified with methanol. Pectinesterase (syn. pectase), which is one of the hydrolytic enzymes effectively inhibited by the material that is the subject of this invention, removes the methoxyl group from pectin to produce pectic acid. Polygalacturonase (commonly called pectinase), which hydrolyzes the glycosidic bonds of pectic acid, and is considered one of the primary enzyme systems for cucumber softening, is also effectively inhibited by the material that is the subject of this invention. Cellulosic substances are essentially long chain polymers of B-D-glucopyranose and are considered to be the most abundant of organic substances in plants. When these polymers are broken down by cellulase and cellulolytic enzymes, the 1,4-glycosidic bonds are split. The cellulolytic enzymes causing this reaction are also effectively inhibited by the material that is the subject of this invention.

An important source of the particular hydrolytic enzymes that are undesirable in the brining process for pickles is very likely the mold-laden cucumber flowers attached to the cucumbers (*Cucumis sativus*) being processed. The cucumber flowers are unavoidably introduced into the brining operations along with the cucumbers. The softening of cucumbers is reputed to be responsible for monetary losses to the pickle manufacturers of upwards of a million dollars annually. Other brined and salted fruits and vegetables have hydrolytic enzymes introduced through various means and they too experience softening spoilage.

It is known that certain plants contain naturally occurring substances and that extracts thereof function as inhibitors for hydrolytic enzymes such as the pectinolytic and cellulolytic enzymes. An impressive list of plants, whose parts (generally the leaves) contain a material that acts as an enzyme inhibitor for the pectinolytic and cellulolytic enzymes, has been collected. The leaves of grape,

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persimmon, dogwood, blueberry, sericea, blackberry, raspberry, and rose are presently considered fairly good sources for hydrolytic enzyme inhibiting substances.

We have now discovered that the forage plant sericea (*Lepedeza cuneata*) is, under certain quite special conditions, not only an excellent source of a material that acts as an enzyme inhibitor for pectinolytic and cellulolytic enzymes, but further we have discovered that when this enzyme inhibiting material is water extracted and/or suitably concentrated from the forage plant sericea, it may be used to advantage in the fermentation operations attendant to the production of brine cured cucumbers. We have also discovered that the presence of the enzyme inhibiting material in sericea is seasonal and limited to about a 10-week period within the growing season. Perhaps even more surprising and unexpected than the circumscribed seasonal occurrence of the hydrolytic enzyme inhibitor in sericea, is our discovery that during the period when the inhibiting material is present in sericea, it (the inhibiting material) is extremely unstable. If special precautions and the exercise of particular techniques for harvesting the plant are not adhered to, the enzyme inhibiting material will quickly disappear entirely from the leaves of the harvested plant.

The graph (FIGURE 1) illustrates how critical the harvest time is for sericea, when the stalks and leaves thereof are to be utilized for the extraction of an enzyme inhibiting material. The data from which the graph (FIGURE 1) was constructed shows that the optimum harvest period for yield of enzyme inhibitor extends from mid-June to the first of September with peak concentration occurring during the latter part of July. Sericea grown in different localities may well exhibit a slightly different optimum harvest period. Furthermore, an altered optimum harvest period might also result from abnormal growing conditions (i.e., plants raised in an artificial environment). However, using well-known techniques to establish the presence of and measure the potency of the enzyme inhibitor, it is a simple matter to check, periodically, for presence of and for the concentration of the inhibitor. Thus, optimum harvest time for any particular set of growing conditions can easily be determined.

We establish the presence and potency of the enzyme inhibitor in plant materials, by use of the following procedure: approximately 20 grams of shredded plant material is macerated in a laboratory blender together with 400 ml. of distilled water. The blending is carried out for a period of 3 minutes following which period of time the resultant slurry is pressed through several layers of cheesecloth. The so strained extract is then clarified by centrifugation for 15 minutes at 3000 r.p.m. The supernatant liquid from the extracted, strained, and centrifuged material is employed for the enzyme inhibitor tests. Commercial enzymes, pectinase and cellulase, are available and are useful for testing inhibitor potency.

**Enzyme solution.**—A new enzyme solution must be prepared each day as described below. A weighed sample (.100 g.) of a commercial pectinase was made up to 100 ml. with water. An aliquot of this solution was diluted to a concentration of 0.1 mg./100 ml.

**Crude enzyme (pectinase and cellulase) preparation.**—A crude enzyme solution can be prepared from cucumber flowers, preferably from the early season's cucumbers. The solution was prepared by blending 20 g. of flowers with 400 ml. of 2% NaCl solution for a period of 3 minutes. The mixture was filtered, and the filtered extract was dialyzed for 3 hours in cellophane tubing against tap water followed by 1 hour against distilled water. The clear, crude solution from cucumber flowers can be used for pectinase and cellulase and can be preserved by storage at 40° C. with a few drops of toluene.

**Enzyme substrates.**—For pectinase enzyme, 6 grams of

sodium polypectate (SP) was dissolved in 500 ml. of 0.02 M NaOH-citric acid buffer at pH 5.0 and 55° C. by mixing in a laboratory blender. The resulting solution was filtered through several layers of cheesecloth and preserved using 1 ml. of toluene. For cellulase enzyme, a 1.2% sodium carboxymethylcellulose (CMC-70 M) solution in sodium hydroxide-citric acid buffer solution at pH 5.0 was used. This solution was prepared in the same manner as the sodium polypectate solution, except 6.0 grams of CMC were used in place of the 6.0 grams of SP.

*Measuring enzyme and inhibitor activity.*—The viscometric method was employed and measurements were taken after a reaction time of 1, 2, and 20 hours. Using 100 units of pectinase activity as equivalent to a 50% viscosity loss in 20 hours of reaction time, a table was set up relating loss in viscosity to units of pectinase activity. This table was calculated from a curve relating the log of pectinase activity units to the percent loss in viscosity. The same general procedure as outlined above for the pectinolytic test is followed for the cellulolytic enzyme test, except that a 1.2% solution of sodium carboxymethylcellulose (CMC) is substituted for the pectate (SP) solution. Standard enzyme activity (control) was measured by mixing enzyme solution with water (2:1 v./v.) and using 1 ml. of this mixture added to 5 ml. of substrate at 30° C. in an Ostwald-Fenske viscosity pipette (uncalibrated, No. 300). Inhibitor activity was measured by substituting one volume of the inhibitor solution prepared as described above for the water. The enzyme and inhibitor were mixed at least 15 minutes before the viscosity measurement was started. Correction was made for the flow time for pure water for each pipette.

As noted earlier, certain precautions must be exercised during the harvest of sericea if the leaves and stems are to be used as a source of enzyme inhibiting material. The sericea leaves, during the harvesting and subsequent storage and before extraction of the inhibitor material, must not be bruised, crushed, or in any way injured until such time as they (the leaves) are being prepared for extraction at the outset of the process for isolating and concentrating the inhibitor material. We have discovered, for example, that sericea cut during the optimum harvest period as for conventional harvest and processing will lose appreciable amounts of the potency of its enzyme inhibitor material in a matter of 15 to 20 minutes and in any event we have found that 6 hours is about the upper limit of holding time for harvested sericea if processing for the extraction of enzyme inhibitor material is contemplated. Furthermore, the leaves of sericea subjected to conventional forage crop harvest methods together with hauling after harvest to a processing center, will upon arrival at the last named location, be devoid, or have only small traces, of the enzyme inhibitor material. We have, therefore, found it essential at the appropriate harvest time, to cut the sericea stalks immediately above the ground, exercising care not to injure the leaves in any manner whatsoever, forthwith to place the stalks and attached leaves in a plastic bag. Placing the plastic bag and contents in cold storage with Dry Ice until such time as the harvested stalks and leaves can be removed from the growing area did not cause the inhibitor to be appreciably retained in the plant material. However, refrigerated storage at 0° F. and below of stalks and leaves sealed in plastic bags can extend enzyme inhibitor potency for several months without significant loss.

The data summarized in Table I clearly reflect importance of harvest and storage methods if the forage plant leaves are ultimately to be extracted for inhibitor ingredient.

Isolation of the enzyme inhibitor material from sericea is accomplished by simple water extraction of the leaves and stems (ethanol and methanol are equally operable as extractants). Following extraction of the inhibitor

material from the leaves, the active principle is complexed and precipitated with an aqueous solution of caffeine and the caffeine complexed material physically separated from the aqueous media and any water soluble extraneous material. The caffeine-inhibitor complex is slightly soluble in water and also exhibits properties of inhibiting hydrolytic enzymes; however, for best results the caffeine should be removed. The caffeine complexed active principle is thereupon resuspended in water and separated from its caffeine complex by liquid-liquid extraction using a water immiscible solvent (ether and chloroform are each operable) after which the extraction separated active principle (in the aqueous layer) is concentrated and subsequently freeze dried.

The enzyme inhibitor material which is the subject of this invention is characterized chemically into a class of compounds called "Leucoanthocyanidins." The isolated inhibitor substance is further classified under the more general term "Flavonoids" and differs in molecular structure from the many flavonoid derivatives by the flavan-3,4-diol structure. The hydrolytic enzyme inhibitor, isolated from sericea, is a light, almost colorless powder which is very soluble in water, methanol, ethanol, and propanol, and is insoluble in ethyl acetate, acetone, benzene, ether, chloroform, acetic acid and strong mineral acids. We would point out, however, that when sericea is harvested for feed purposes by conventional means, cut mechanically or chopped for dehydration as contrasted with our particular prescribed harvest methods, the inhibitor activity disappears almost completely although the sericea still exhibits a positive test for the presence of tannins. Thus, the tannin content of sericea does not necessarily correlate with content of the inhibitor that is the subject of this invention.

The following example illustrates one method for isolating and concentrating the enzyme inhibiting material:

A 250-gram quantity of fresh sericea leaves harvested at the appropriate time and with the exercise of the prescribed precautions as noted above, or alternatively, 250 grams of sericea leaves harvested and stored under refrigeration in plastic bags as described above, was blended together with 2 liters of distilled water for a period of three minutes in a one-gallon capacity laboratory blender. The resulting slurry was filtered through a large Buchner funnel using several thicknesses of cheesecloth as the filter medium. The above steps of blending and filtering were repeated ten times so that ultimately a total of 2500 grams of sericea leaves was extracted with the result that 18 liters of filtered sericea leaf extract was obtained. The filtered leaf extract was centrifuged to remove suspended plant materials, the clarified solution cooled to 15° C. and again centrifuged. To this twice centrifuged 15° C. leaf extract 6 liters of cooled (10 to 12° C.) caffeine solution (15 g./l.) were added slowly and with constant stirring. The mixture of leaf extract and caffeine solution was then cooled and held at a temperature of from 8 to 10° C. until precipitation of the complex was complete. The caffeine inhibitor complex was allowed to settle for approximately 4 hours but the complex can be held in water suspension overnight at 15° C. The supernatant liquor, after the complex had settled, was then centrifuged to remove additional quantities of the suspending liquor. The complexed material can be utilized as such as a hydrolytic enzyme inhibiting material, or may be freeze-dried for use later. Following centrifugation, the caffeine complex was resuspended in approximately 2 liters of water and the complex in suspension then subjected to liquid-liquid extraction with chloroform (diethyl ether is equally operable) for a period of 20 hours. Following extraction, the water layer was removed from the extractor, filtered through paper, and placed in a vacuum evaporator where the total volume was reduced by approximately 1/2 (water bath temperature about 50° C.). The reduced volume of inhibitor solution was then divided into two equal portions, each portion placed in a 2-liter

