To the Graduate Council:

I am submitting herewith a thesis written by Jeffrey Lee Womack entitled "Microbial and endogenous polygalacturonase activity in broccoli during storage." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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Dean of The Graduate School

Microbial and Endogenous Polygalacturonase Activity in Broccoli During Storage

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Jeffrey Lee Womack
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Dedication

This thesis is dedicated to my mother

Ms. Shirley Ann Womack

who has given me love and support throughout my education

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Abstract

Broccoli florets were cut into 3 to 10 g and, washed in a 200 ppm chlorine solution or left unwashed . The florets were packaged and stored at 7 and 13°C for a 16-d period. The initial washing reduced the microbial counts by 2 log CFU/g on the florets. After 16-d storage at 7 and 13°C, there were significant differences in microbial counts between washed and unwashed broccoli ($P>0.05$). There was a greater increase in microbial counts on broccoli stored at 13° C, 3.5 log CFU/g, than on broccoli stored at 7°C, 2.5 log CFU/g. Temperature had more effect on controlling microbial growth over the 16-d period than washing the broccoli. Endogenous polygalacturonase (PG) activity in the broccoli did not increase during16-d storage at 7, or 12-d storage at 13° C. However, PG increased for the broccoli stored 16-d at 13° C ($P<0.05$).

Endogenous PG activity of broccoli florets stored at 13°C did not increase during an 8-d storage period. Internal broccoli tissue did not soften for the 8-d period ($P>0.05$). A method for measuring enzyme activity due to PG produced by spoilage microorganisms was developed. Total PG activity levels increased during 8-d storage indicating increased microbial activity.

Broccoli cores softened after 4-d storage at 13°C in broccoli medium that was inoculated with indigenous broccoli aerobic spoilage microorganisms. At that time, aerobic microbial counts in the medium were 7.5 log CFU/g. Broccoli cores stored in sterile broccoli medium did not soften after 4-d. This would indicate that PG produced by the high level of microorganisms has a softening effect on the broccoli texture.

Table of Contents

List of Tables

Table

Table

Chapter 1

Introduction

Minimally processed refrigerated (MPR) fruits and vegetables have become one of the fastest growing markets in recent years. An increased emphasis on quality and convenience has brought about this change (King and Bolin, 1989). Minimal processing of fruits and vegetables consists of unit operations such as, washing, sorting, peeling, and slicing (Rolle and Chism, 1989). After processing, MPR products still have living, respiring tissues which contribute to their fresh-like quality (Wiley, 1994). Due to the continuing respiration of the plant, extension of shelf life becomes a major concern. Chemical reactions that can lead to undesirable quality changes in the product take place (King and Bolin, 1989). The cut surfaces on the product are prime areas for microbial growth. The presence of these microorganisms not only may make the product unsafe, but may also contribute undesirable quality changes (Wiley, 1994).

Quality changes that may occur due to chemical changes and microbial growth include a loss of texture and color loss (often browning). Softening of the plant product is the most common texture problem in MPR products, and is a major problem in MPR lettuce, celery, cauliflower, and broccoli. In broccoli, softening contributes as much as 30-40% crop loss in some regions of the world (Hildebrand, 1989). The texture breakdown can be attributed to pectinolytic enzymes. These enzymes degrade the pectic substances that provide rigidity to the plant, and as a result, softening of the plant tissue occurs (Wiley, 1994).

Polygalacturonases (PG)(EC 3.2.1.15) can be found in higher plants and are produced by various species of microorganisms. PG may contribute considerably to texture loss. Wounding of plant tissue during processing allows the PG in the cell walls to be released and to react with substrate. PG hydrolyzes polygalacturonic acid forming galacturonic acid by-products (Whitaker, 1994). PG is produced by two main types of microorganisms associated with spoilage of broccoli (Hildebrand, 1989). The PG enzymes produced by *Pseudomonas* spp. and *Erwinia* spp. along with the endogenous PG are the contributing factors for broccoli tissue softening (Hildebrand, 1989).

Several studies have been conducted by microbiologists investigating PG and its activity associated with microorganisms such as *Pseudomonas* and *Erwinia*. Studies also have been conducted in the area of postharvest physiology related to the effects of endogenous PG on many fruits and vegetables. Researchers in both areas use enzyme assays to determine the amount of PG activity produced by the microorganisms or found in the vegetables. Very little research has been conducted joining the areas of microbiology and postharvest physiology in order to determine the effects of PG produced by microorganisms and endogenous plant PG on tissue softening. Three studies were performed in order to relate tissue softening of broccoli to PG produced by spoilage microorganisms and PG endogenous to the broccoli. The objective of study 1 was to determine the effect of washing with 200 ppm chlorine and storage temperature on microbial growth and endogenous PG activity. Study 2 was performed to determine the amount of endogenous PG activity in broccoli and the texture breakdown of broccoli due to endogenous PG. Also study 2 was used to evaluate a method for measuring microbial PG activity together with endogenous PG. The objective of study 3 was to determine the

effect of PG produced by broccoli spoilage microflora on texture of broccoli. These three studies on PG activity in or on minimally processed broccoli, will provide a better understanding of factors that contribute to softening and overall quality of broccoli.

Chapter 2

Literature Review

Minimal processing is defined by Shewfelt (1987) as "handling, preparation, packaging, and distribution of agricultural commodities in a fresh-like state." Minimally processed refrigerated (MPR) fruits and vegetables have been one of the fastest growing markets in the food industry. The European countries were among the first to make strides in the MPR area. The MPR market in Europe increased from 400 tons in 1985 to 35,000 tons in 1989 (Nguyen-the and Carlin, 1994). Minimal processed lettuce was one of the first commodities in the United States (King and Bolin, 1989). Minimally processed lettuce increased from 140 million lb. in 1984 to 500 million lb. in 1987 (King and Bolin, 1989). Fresh Express, the largest manufacturer of minimally processed salad, showed an increase in annual sales from $189 million in 1995 to $304 million in 1997 (Internet, 1996).

The steady emergence of MPR fruits and vegetables can be attributed to the consumer desire for nutritious, high quality, fresh products (Shewfelt, 1987; King and Bolin, 1989). Retail food stores, restaurants, carry-out establishments, and commissary units have utilized the convenience that MPR products provide (Wiley, 1994). The involvement of these large industries has resulted in an array of products used for minimal processing. Most fruits and vegetables, including strawberries and broccoli, can be minimally processed (Wiley, 1994).

MPR fruits and vegetables are in a raw, fresh state where physiological changes, which can affect quality, still take place . Wounding of the plant tissue from

processing, temperature abuse, or improper handling may induce physiological changes (Wiley, 1994; Rolle and Chism, 1987). In fruits and vegetables, respiration rate increases after minimal processing, resulting in rapid deterioration of the product. Ethylene production in broccoli increases, causing color defects such as pigment loss and yellowing of the product (Watada et al., 1990). Under stress conditions, enzymes are released from their cellular compartments. These enzymes then react with substrates causing changes in texture, color, and flavor (Rolle and Chism, 1987). Damaged surfaces on the plant allow leakage of nutrients which microorganisms can utilize for rapid growth (Nguyen-the and Carlin, 1994; Wiley, 1994). The microbial and chemical changes result in an overall quality loss of the product.

Broccoli

Broccoli (*Brassicas oleracea* var. *italica*) was first introduced to the United States by Italian immigrants (Buishand et al., 1996). In recent years, broccoli has increased in popularity due to its nutritional composition (Table 1) and health benefits. The carbohydrate portion is mainly fiber compounds including pectin. Broccoli is classified as a flower or flower-bud, along with cauliflower (Burton, 1982; Kays, 1991). Flowers are made of compressed shoots that contain foliar parts used in reproduction (Kays, 1991). Flowers differ in size, structure and longevity and become highly perishable when detached from the parent plant (Kays, 1991). Minimal processing increases the rate of many physiological changes, causing broccoli to become a highly perishable product (Simons, 1987).

Table 1-Nutritional composition of broccoli

Components	Amount
Water	89.1%
Protein	3.6%
Fat	0.3%
Carbohydrate	5.9%
Calcium	103 mg
Phosphorus	78 mg
Iron	1.1 mg
Sodium	15 mg
Potassium	382 mg
Magnesium	24 mg
Vitamin A	2,500 IU
Thiamin	0.10 mg
Riboflavin	0.23 mg
Niacin	0.90 mg
Vitamin C	113 mg

(Salunkhe and Deshpande, 1991)

Respiration

During respiration, energy is released through the breakdown of carbohydrates, lipids and other substrates (Burton, 1982; Kays, 1991). Glucose and fructose are the two predominant sugars used as substrates during respiration in broccoli (King and Morris, 1994). Polyunsaturated fatty acids and proteins also are used for energy during broccoli respiration (Zhuang et al., 1994). An increase in temperature of the broccoli increases the rate of respiration. At elevated temperatures sugars and proteins rapidly decrease in 2 to 3 days, resulting in low levels of substrate accessible for metabolism (Pogson and Morris, 1997). Respiration of broccoli also results in heat generation (Burton, 1982).

Gas Composition

Gas composition can have both beneficial and detrimental effects on broccoli quality. Oxygen stress occurs when there is a decrease in the amount of oxygen present in the plant cells (Kays, 1991). The lack of oxygen results in a decrease in energy yield. Respiration is increased in order to maintain energy levels (Kays, 1991). The increased respiration results in the loss of chlorophyll in the broccoli tissue (Kays, 1991).

Carbon dioxide (CO_2) concentrations in the surrounding atmosphere cause changes in broccoli quality. CO_2 levels in the range of 6 to 10% have a beneficial effect on broccoli. CO_2 slows the rate of respiration, prevents yellowing of the flower, and reduces the loss of chlorophyll (Izumi et al., 1996; Bastrash et al., 1993). Moderate CO2 levels can also help retain nutritive value of broccoli by maintaining ascorbic acid content of broccoli (Barth et al., 1993).

Ethylene

Minimal processing of broccoli increases the production of ethylene in the tissue (King and Morris, 1994). Ethylene is a growth hormone that affects the metabolic rate of plants at low concentrations (Kays, 1991). Rapid increases of ethylene production can be seen in the first six hours after processing (King and Morris, 1994). Excessive ethylene production increases respiration rate, alters enzyme activity, increases membrane permeability, and decompartmentalizes cells (Burton, 1982; Kays, 1991). These alterations affect broccoli quality by degrading chlorophyll, accelerated yellowing, and softening texture (Kays, 1991).

Enzyme activity

Wounding of plant tissue causes disruption of the plant cells (Wiley, 1994), which allows enzymes and substrates in the plant to react (Schwimmer, 1972).
Polyphenol oxidase enzymes, when released, react with substrates and cause browning (Wiley, 1994). Cystine lyase enzymes break down cystine to produce ammonia and sulfur off-odors in broccoli (Lim et al., 1989). Pectinesterase (PE) and polygalacturonase (PG) react with pectin and pectic acid causing softening of broccoli (Kays, 1991).

Modified Atmosphere

Modified atmosphere packaging (MAP) has been one of the most successful ways of extending quality shelf-life of highly perishable products (Wiley, 1994). During respiration, fresh produce will naturally modify the atmosphere in a package (Ballantyne, 1988). Packaging materials must be designed to allow for an equilibrium of O_2 and CO_2

(Ballantyne, 1988). Small or large amounts of O_2 or CO_2 can result in quality changes in the plant material (Kays, 1991). In studies by Izumi et al. (1996); Bastrash et al. (1993); Makhlouf et al. (1989), and Barth et al. (1993) it was found that a 2% O_2 concentration and a 8% CO_2 concentration, combined with a 1°C and 95% RH storage, could extend the quality and shelf-life of broccoli up to 5 to 7 weeks. Although modified atmosphere can help maintain broccoli quality, it does not have a significant effect on microbial populations (Mohd-Som et al., 1993).

New techniques for maintaining broccoli quality have been investigated in recent years. Studies by Gillies and Toivonen (1995) and Toivonen (1997) revealed that rapid hydro-cooling, as opposed to traditional top-icing, postharvest improves color retention and maintains firmness in broccoli. As shown by Tian et al. (1997) and Forney (1995), hot water dipping of broccoli postharvest prevents yellowing and delays senescence. Barth et al. (1992) revealed that misting broccoli at different time intervals during storage helps maintain color, texture, and vitamin C.

Pectin Characteristics

Carbohydrates (90%) and protein (10%) make up the primary walls of plant cells. Pectin, cellulose and hemicellulose form the carbohydrate portion of the primary cell walls (Mohnen et al., 1996). Pectin is a generic term for the group of pectic substances that form sugar-acid gels (Whitaker, 1994). Homogalacturonon, rhamnogalacturonon I (RG-I) and rhamnogalacturonon II (RG-II) form the polysaccharide family of pectin's (Pellerin et al., 1996).

Pectins are imbedded throughout the cell wall and are the most abundant in the middle lamella region (DellaPenna et al., 1996). Pectins play a major role in providing the cell wall with strength, ion exchange and sieving properties, adhesion, and communication (Mohnen et al., 1996). RG-I, RH-II, and homogalacturonon all contain galacturonic acid (Mohnen et al., 1996). RG-I is a large branched chain of polysaccharides with a backbone consisting of a repeating disaccharide of 2-alpha-L-rhamnosyl-1,4-alpha-D-galactosyluronic acid (Mohnen et al., 1996). RG-II contains a homogalacturonon backbone of nine 1,4 linked alpha-D-galactosyluronic acid residues (Albersheim et al., 1996). Homogalacturonons are chains of alpha-1,4-linked D-galacturonic acid (Mohnen et al., 1996).

Pectic Enzymes

Pectic enzymes are enzymes compartmentalized in the cell walls of higher plants and in microorganisms (Whitaker, 1994). Pectic enzymes are deteriorative enzymes in that they breakdown pectin and pectic substances resulting in softening of fruits and vegetables (Whitaker, 1994). PG, PL, and PE are the three main pectic enzymes or pectinases. PG and PE are found naturally in healthy plants and are also produced by numerous strains of microorganisms. PL are only produced by microorganisms (Whitaker, 1994). The pectic enzymes are separated on the basis of substrate specificity, by method of polymer split (cleavage), and by site of the split.

PE (EC 3.1.1.11) hydrolyzes the methyl ester groups of galacturonic acid residues in the pectic chain, resulting in the formation of galacturonic acid groups and methanol.

Pectinesterases can attack and remove methoxyl groups anywhere along the pectin chain (Whitaker, 1994). The optimal pH for activity is at 7.5, however, the optimum pH changes with concentration of salts. At high concentrations of salt and a pH of 6.0 activity is inhibited. Compounds such as formaldehyde, pyridine and quinoline also inhibit activity (Kertesz, 1951). PE in higher plants has been found to be heat resistant up to approximately 55°C. At temperatures above 60°C some inactivation of the enzyme occurs (Kertesz, 1951).

PL (EC 4.2.2.2) are enzymes associated with soft rot produced by microorganisms such as *Erwinia spp.* and *Pseudomonas spp.* PL cleave galacturonic acid by transelimination of hydrogen from the 4 and 5 carbons of galacturonic acid (Whitaker, 1994). PL are subdivided on the basis of preference for pectin or pectic acid as substrate. They also are further classified into exo and endo splitting (Whitaker, 1994). PL have a pH optimum of 8.5 to 9.5. The presence of calcium is required for PL activity (Whitaker, 1994). Activity of PL is inhibited by the presence of phenols and quinones (Bateman and Millar, 1966).

PG (EC 3.2.1.15) are divided into the subgroups of endo or exo polymethylgalacturonases and endo or exo polygalacturonases (Whitaker, 1994). The two subgroups are divided on the basis of substrate specificity and method of attack. Polymethylgalacturonases prefer pectin as a substrate and PG prefer pectic acid as a substrate (Whitaker, 1994). PG are found naturally in higher plants and are produced by the soft rot species of *Erwinia* and *Pseudomonas*. Maximum activity is achieved for PG in a pH range of 4.5 to 6.0 and at temperatures from 30°C to 50°C. Temperatures of

80°C to 100°C cause the enzyme to become inactive (Kertesz, 1951). Calcium increases activity of PG but its presence is not required (Whitaker, 1994).

PG and PL are the main deteriorative enzymes associated with the undesirable texture quality changes in minimally processed fruits and vegetables. PG and PL have been associated with the soft rot of lettuce, celery, cabbage, cauliflower, and broccoli (Albrecht et al., 1995). Soft rot or head rot is a major concern in the broccoli producing industry. Crop loss can exceed as much as 30-40% in some regions of production (Hildebrand, 1989). Pectic enzyme producing species of *Erwinia* and *Pseudomonas* have been suggested as contributors to soft rot.

Enzyme activity

Measurement of enzyme activity is a complicated procedure due to sensitivity of enzymes. Factors such as pH, temperature, and inhibitors make obtaining consistent results difficult. Enzyme extraction, purification, and activity assays are the basic steps involved in order to measure enzymes. However, each one of these steps varies depending on the type of material, type of enzyme, and assay procedure.

The extraction process involves homogenizing the material that contains the enzymes. The homogenized material is then filtered to remove impurities. The types of material used for filtering and the number of filtration times vary. Enzymes then are extracted using an extraction fluid. The choice of extraction fluid depends on whether the enzyme is soluble or membrane bound (Whitaker, 1994). Salt solutions with ionic strengths in the range of 0.1 to 0.5 are used for detachment of the enzyme from the cell wall. Sodium chloride is the most common salt used in the removal of PG from the cell

wall (Gross, 1982; Jen and Robinson, 1984; Miller et al., 1987). The pH of the salt solution is then adjusted and maintained in the range of 6.0 to 7.5. The solution is buffered in order to protect the enzymes from the acids released when the cell wall is ruptured (Whitaker, 1994). The amount of extraction fluid used is usually two to three times the volume of the tissue, but the extraction time varies. In PG extraction, times have varied from 1h to 24h (Jen and Robinson, 1984; Abu-Sarra and Abu-Goukh, 1992). After the enzymes have been extracted from the tissue, centrifugation is used to remove the cellular debris (Whitaker, 1994). The centrifugation speeds range from 9,000 to 27,000 x g depending on the type of plant tissue. Times for centrifugation vary from 5 to 30 min. These procedures are usually carried out at temperatures near 0°C to ensure enzyme stability (Whitaker, 1994). In PG extraction, 4°C is the most common temperature used (Gross, 1982; Jen and Robinson, 1984; Miller et al., 1987).

Purification of enzymes must be done after the extraction procedures have been performed. In the purification step, the desired enzyme is separated from proteins, pigments and other compounds contained in the extraction solution (Whitaker, 1994). Enzymes can be separated on the basis of size, change in ionic strength, pH, or dielectric constant of the eluting solvent (Whitaker, 1994). These techniques can be performed using different types of chromatography, such as ion exchange and gel filtration. In most studies involving PG purification, gel filtration is used (Gross, 1982; Jen and Robinson, 1984). Gel filtration separates compounds based on size. Filtration columns are prepared using materials such as Sephadex gels which vary in type depending on molecular weight separation range (Whitaker, 1994). An elution buffer is used to elute the extraction

solution through the column where enzyme extracts are collected based on a predetermined void volume.

A spectrophotometric method based on following the rate of conversion of substrate to product is most commonly used for activity assays. Methods by Nelson (1944) and Gross (1982) are commonly used for analysis of PG activity. Both methods are based on measuring the rate of formation of reducing groups by observing changes in absorbance at a set wavelength (Nelson, 1994; Gross, 1982). In PG assays, a reaction buffer containing polygalacturonic acid is reacted with the enzyme sample. The reaction temperature commonly used for optimum PG activity is 30°C. However, reaction times vary from study to study (Gross, 1982; Jen and Robinson, 1984; Miller et al., 1987). After the proper reaction time has elapsed, the reaction is stopped by inhibiting enzyme activity by either adding cold copper reagent (Nelson, 1944) or a cold borate buffer (Gross, 1982) to the reaction mixture. Color reagents are then added to the solution to form a color that can be measured at a given wavelength. The Nelson (1944) method involves boiling the mixture in water bath for 20 min and then immersing the mixture in an ice bath. The arsenomolybdate color reagent is added, the samples are mixed and centrifuged, and absorbance is then at 540 nm. In the method by Gross (1982), 2-cyanoacetamide is added to the samples. The samples are boiled for 10 min, to form the color, and the absorbance is read at 276 nm. The method by Gross is less complicated than the Nelson (1944) method because it requires the addition of only one reagent and does not require a centrifugation step.

Microbiology

Minimally processing of vegetables such as broccoli involves steps in which the plant tissue is wounded. The processing not only allows for physiological change to take place but also microbial changes (King and Bolin, 1989). The wounded surfaces of the plant tissue release water and water soluble nutrients. The natural microflora use these nutrients to grow and spread (Adams et al., 1989; King and Bolin, 1989). Loss of textural quality characteristics can be attributed to microbial growth. Soft rot is the major result of bacterial decay caused by *Erwinia* spp. and *Psuedomona* spp. (Hao and Brackett, 1994), which produce PL and PG (Hao and Brackett, 1994; Collmer and Keen, 1986).

Erwinia spp. are Gram-negative, non-sporulating, motile bacteria that grow in aerobic and anaerobic conditions. *Erwinia* spp. have been isolated from a wide variety of vegetables including potatoes, cabbage, lettuce, celery, and broccoli (Jay, 1992). *Erwinia carotovera* is the primary strain for producing pectin degrading strains of enzymes, PL and PG (Lund, 1983).

Pseudomonas spp. are Gram-negative, non-sporulating, motile rods that grow in aerobic conditions (Lund, 1983). Species of *Pseudomonas* have been isolated from vegetables such as celery, asparagus, cauliflower, and broccoli. *Pseudomonas fluorescens* and *Pseudomonas marginalis* have been identified as the most important bacteria associated with broccoli head rot (Hildebrand, 1989). Both species of *Pseudomonas* produce PG and PL. These enzymes are exported from the bacterial cytoplasm to the host tissue. The enzymes are active and cleave the pectic polymers in the primary cell wall and middle lamella of the host plant. The cleaving of the pectin

results in the loss of plant tissue rigidity or what is called soft rot (Collmer and Keen, 1986).

Chapter 3

Materials and Methods

Study 1 - Washing treatment and storage temperature effects on broccoli

Broccoli Preparation

Broccoli was shipped from Mann's produce, California and purchased from Neel's Produce, Knoxville, TN. Cases of broccoli were transported to the Department of Food Science and Technology and held at 4°C until processed.

Broccoli Processing

Broccoli stalks were manually cut into florets ranging in size from 3 to 10 g. Approximately 3,000 g of broccoli was mixed so florets from the same stalk were randomly dispersed. The broccoli was then divided into 1,500 g lots.

A dip solution was prepared by mixing 8,000 ml of tap water with 32 ml of sodium hypochloride (5.25%). The dip was chilled to 7°C using 2,000 ml of ice water to maintain the broccoli florets at a constant temperature and to keep the chlorine in solution. A batch of broccoli florets (1,500 g) was dipped into the chlorine solution for 60 s. The broccoli was then removed from the dip, drained and rinsed using 8,000 ml of tap water. The other 1,500 g of broccoli florets were not washed and were considered to be a control.

Packaging and Storage of Broccoli

The unwashed and washed broccoli was packaged in Cryovac PD-941 broccoli bags, obtained from Cryovac-Sealed Air Corporation (Duncan, SC). The bags were modified from an original 61 cm x 36 cm size to 18 cm x 20 cm. For each treatment, 14 bags were each filled with 100 g of broccoli florets and heat sealed.

Seven bags of broccoli samples from each treatment were stored at 7°C, and seven at 13°C. The samples were analyzed for enzyme activity and microbial analysis after 0, 2, 4, 6, 8, 12, and 16 days of storage.

Microbial Analysis

A 25 g sample of broccoli was removed from each bag for microbial analysis. The sample was placed into a stomacher with 225 ml of 0.1% peptone water and pummelled for 2 min at high speed in a Seward model 400 stomacher (Teckmar, Cincinnati, OH). Serial dilutions were made in 0.1% peptone water, and surface plated onto tryptocase soya agar (TSA) (Difco) for enumeration of aerobic microflora (APC) and *Pseudomonas* C-F-C selective agar (Unipath-Oxoid) for enumeration of *Pseudomonas* spp. TSA plates were incubated at 32° C for 48-h and C- F- C plates were incubated for 48-h at 30° C. All microbial counts were expressed as log CFU/g.

Polygalacturonase Analysis

Sample Preparation

Samples of 50 g of broccoli were taken from each bag of broccoli. Each 50 g sample was blended with 100 ml of distilled water in a Waring Blender at high speed for

1 min. Subsequently, the homogenate was filtered through 6 layers of grade 40 cheesecloth (Fisher Scientific). The residue was rewashed in 100 ml of distilled water. The suspension was refiltered and the residue was suspended in 50 ml of 1N NaCl (pH 6.0). The mixture was stirred in a 100 ml beaker placed on a magnetic stir plate for 3h at 4°C. After 3h, the slurry was filtered through 6 layers of cheesecloth and stored at 4°C for 24-h. After storage, the filtrate was centrifuged in a Beckman J2-HS centrifuge at 9,000 x g for 15 min. The supernatant was decanted and recentrifuged at 9,000 x g for 15 min. A 10 ml sample of supernatant was collected and held until further purification.

Purification

Gel filtration columns were prepared by mixing Sephadex G-25 fine mesh gel with 50 mM Na-Acetate buffer (pH 6.2). The column packing procedure was performed according to methods by Spencer (1977). A marker of Blue Dextran 2,000 was eluted through the column in order to determine void volume. A void volume of 2 ml and a collection volume of 3.5 ml was determined and used consistently throughout the study. A 1.5 ml sample of extract was eluted through the desalting column, and 50 µl of the desalted extract was used for enzyme assays. Recovery efficiency of the desalting columns was determined by eluting a 0.02% solution of purified pectinase (Sigma) through the columns and collected according to the void and collection volumes. This procedure was repeated every day of desalting.

Enzyme Assays

The 50 µl sample of the purified extract was mixed in with 150 µl of reaction buffer consisting of 0.2% polygalaturonic acid (Sigma) mixed with 37.5 mM Na-Acetate buffer (pH 6.2). The tubes containing the reaction mixtures were incubated in a 30°C ISOTemp 220 water bath for 3h. After 3h the reactions were stopped by adding 1 ml of 100 mM cold borate buffer (pH 8.0 stored at 4°C). A 0.2 ml volume of 1% 2-cyanoacetamide (Aldrich) was added to the reaction mixture. The mixtures were heated in a boiling water bath for 10 min. After removal from the water bath the mixtures were allowed to cool to 25°C (approx. 15 min) before assessment of absorbance at 276 nm using a Shimadzu UV-2101 PC Scanning Spectrophotometer. The blank contained all solutions except the enzyme extract. All assays were performed in triplicate.

Study 2 - Endogenous and microbial enzyme analyses and effects of endogenous enzymes on texture.

Endogenous enzyme analysis was used to determine the activity levels of PG enzymes in broccoli, and the effect they had on broccoli texture. In this study, broccoli was not washed. The broccoli samples were stored at 13°C for 0, 2, 4, and 8 days. Only APC were used for microbial analysis. The enzyme extraction procedures and assays were conducted as described for study 1.

Filtrate collected from the first 3 steps of extraction was collected and assayed to determine the number of microorganisms lost during extraction, and to determine activity of enzymes produced by microorganisms. A sample was taken and analyzed for APC using the procedure described under study 1. Approximately 200 ml of filtrate was

collected and stored at 4°C for 24 h. After storage the filtrate was centrifuged at 9,000 x g for 15 min. The supernatant was decanted and recentrifuged at 9,000 x g for 15 min. After centrifugation, 10 ml of the supernatant was removed, and a 50 µl was analyzed for enzyme content according to the enzyme assay procedure in study 1.

Texture Analysis

Broccoli stalks, removed during broccoli processing, were cut into 5 cm sections. The sections were placed in the bags with the broccoli samples used for endogenous PG analysis. On the day of enzyme analysis, cores were taken from the stalk using a 0.64 cm (¼ in) sterile corer. The cored sections were trimmed to 2 cm length using a sterile knife. Texture of the cores was measured using a TX-12 texture analyzer with a compression attachment. Compression was applied over 2 mm of the core length. Maximum compression force was used as an indicator of broccoli firmness.

Total Enzyme Analysis

A method was developed to determine the total activity (microbial and plant enzymes) of the broccoli samples. Broccoli was prepared and stored under the same conditions used for endogenous activity in this study. APC were used for microbial analysis. The enzyme extraction procedures and assays were conducted as described for study 1 with the exception that the first three steps of the enzyme extraction process were deleted. A 50 g sample of broccoli was ground in 50 ml 1N NaCl for 1 min, and then centrifuged as outlined for endogenous enzyme analysis. Further purification and assay of activity were the same as for endogenous enzyme analysis.

Study 3 - Microbial enzyme activity and effects on texture of broccoli

Enzyme analysis was conducted to determine the amount of PG produced by broccoli spoilage microoganisms at different growth stages, and the effect that microbial enzymes have on texture. A microbial growth medium was prepared by blending a 1:2 ratio of broccoli and water. A pair of 1000 ml flasks was filled with 500 ml of the broccoli meduim. The medium was then capped and sterilized in an autoclave at 121°C for 15 min. The autoclave step was performed to inactivate endogenous enzymes in the broccoli and to produce sterile medium. Mixed strains of broccoli spoilage microorganisms were collected from the liquid of spoiled broccoli that had been stored for 2 weeks at 13°C. The isolated organisms were grown in tryptic soy broth (TSB) (Dilfco) at 32°C. Three 24-h culture transfers were performed on the spoilage microorganisms to allow growth up to approximately 8 log CFU/g. One flask was inoculated with approximately 3 log CFU/ml spoilage microorganisms and the uninoculated flask was used as a control.

A 40 g sample of contaminated broccoli medium was used for analysis of enzymes and 10 g was used for determining the APC. The sample for enzyme analysis was filtered through six layers of cheesecloth, and the filtrate was centrifuged at 9,000 x g for 15 min. The sample was decanted and recentrifuged at 9,000 x g for 15 min. After centrifugation, 10 ml of the supernatant was collected, and 50 µl was assayed using the assay procedure of study 2. The enzyme and microbial analyses were performed daily for up to five days.

Texture analysis

Broccoli stalks taken from the processed broccoli were cut into 5 cm sections. Two matching 0.64 mm cores were taken side-by-side from each section in order to eliminate the influence of plant variation. Each core was trimmed to 2 cm length using a sterile knife. One core was placed in the inoculated broccoli medium and the other in the sterile medium as a control. Each day over the 5-d period, matching cores were removed from the broth and measured according to the texture procedure described in study 2.

Statistical Analysis

Analysis of variance for microbial counts and enzyme activity data collected in study 1 was done by PROC GLM utilizing SAS (1989). Main effects of days of storage, wash treatment, storage temperature and replication with interactions of day*treatment, day*temperature and treatment*temperature were included. Effects were considered significantly different at $P<0.05$. PROC MIXED (SAS, 1996) was used to generate LSMeans for significant effects of interactions.

Analysis of variance for APC, enzyme activity and texture data of study 2 were done by PROC GLM utilizing SAS (1989). Main effects were days of storage and replication. Effects were considered significantly different at $P<0.05$. PROC MIXED was used to generate LSMeans for significant effects.

Analysis of variance for APC, enzyme activity and texture data collected in study 3 were done by PROC GLM utilizing SAS. Main effects were days of storage and replication. Effects were considered significantly different at $P<0.05$. PROC MIXED was used to generate LSMeans for significant effects.

Chapter 4

Results and Discussion

Study 1 - Effect of washed versus unwashed treatments and temperature on enzyme activity

Minimally processed broccoli was washed with a 200 ppm chlorine solution on a day-to-day basis to reduce microbial counts on the surface of broccoli. This procedure was performed to reduce the influence of microbial produced enzymes on the washed broccoli. The days of storage, wash treatment, storage temperature and replications all had significant effects on the APC of the minimally processed broccoli (Table 2). The interactions of the wash treatments with days of storage and with storage temperature were both significant, and these data are shown in Table 2. Due to no growth on the plates at day 0, APC counts could not be determined. However, preliminary research indicated that initial microbial counts were reduced up to two log CFU/g with an initial wash treatment. As can be seen in Table 3, Figure 1, after 2-d storage the APC on washed broccoli stored at 7°C was significantly lower (2 log CFU/g) than unwashed broccoli. However, the APC of washed broccoli stored at 13°C was not significantly lower than that of unwashed broccoli ($P<0.05$). The washed broccoli APC remained significantly lower at 7°C until after day 12. The APC for the washed broccoli is consistent with reports by Faridaii et al. (1995). Faridaii et al. (1995) reported a steady increase in microbial growth on broccoli that was misted over a 5-d period. The initial decrease in APC when broccoli was washed as found in the preliminary research, and at 7°C storage is consistent with results found by Brackett (1987) and Faridaii et al. (1995). However,

Table 2-Analysis of variance of aerobic plate counts (APC) for washed and unwashed Broccoli stored at 7 and 13°C, for a 16-d.

Source	DF	APC mean square	APC Pr>F
Day	5	14.00	0.0001
Wash	1	3.63	0.0001
Temperature	1	24.24	0.0001
Replication	1	4.25	0.0001
Day*Treatment	5	1.09	0.0001
Day*Temperature	4	0.85	0.0008
Treatment*Temperature	1	10.22	0.0001
Error	58	0.15	

Table 3-Aerobic plate counts (APC) for washed and unwashed broccoli for 16-d storage period.

Storage time (days)	Storage temperature			
	7°C		13°C	
	Washed	Unwashed	Washed	Unwashed
2	2.65±0.49 l	4.53±0.59 ij	3.98±0.36 jk	4.39±0.56 ijk
4	3.91±0.92 k	5.60±0.17 gh	5.71±0.22 fgh	5.88±0.67 efgh
6	4.63±0.59 i	5.78±0.18 efgh	6.21±0.17 defg	6.19±0.06 def
8	4.52±0.10 ijk	6.25±0.12 def	7.40±0.28 ab	7.02±0.54 bc
12	5.43±0.64 h	6.29±0.13 de	7.40±0.28 a	7.14±0.71 b
16	6.48±0.81 d	6.52±0.23 cd		

APC determined after incubation at 32°C for 48 h
N=6
Means followed by like letters are not statistically different (P>0.05)

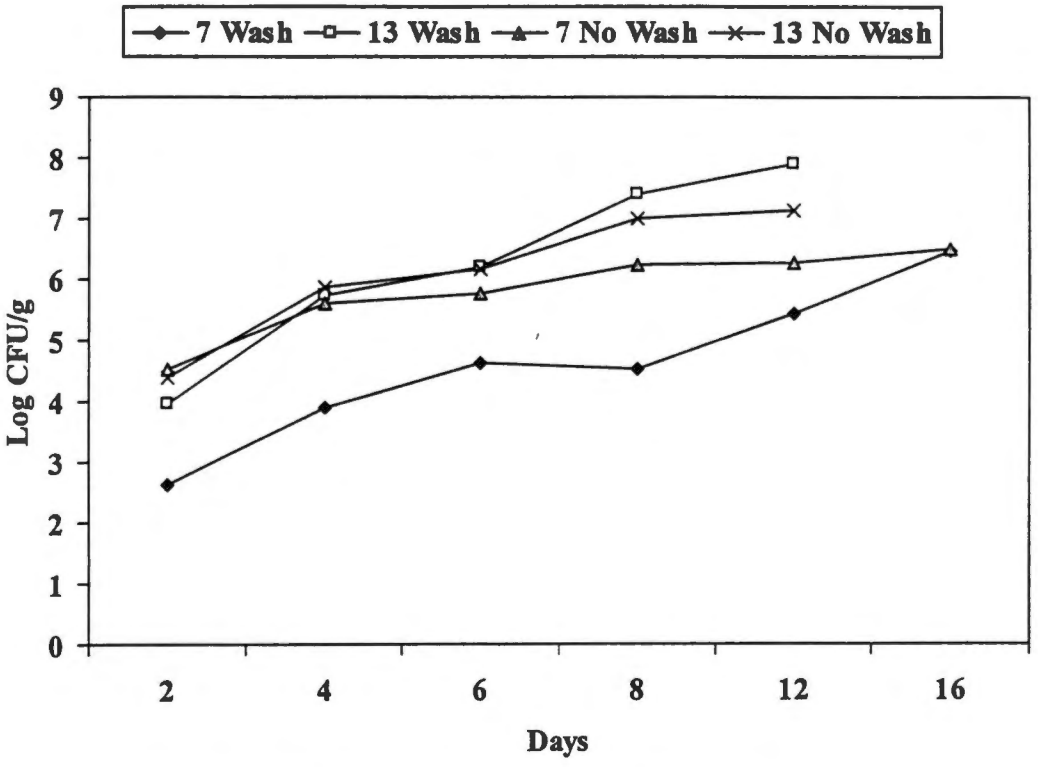

Figure 1. The effect of wash treatments and temperature on aerobic microbial growth over a 16-d storage period.

Albrecht et al. (1995) reported no effect on APC counts when samples were washed with either water or a chlorine solution.

As shown in Table 3, the APC on washed broccoli by day 2 and unwashed broccoli by day 8 stored at 7°C were significantly lower than on broccoli stored at 13°C. Storage of broccoli at 7°C significantly slowed the microbial growth rate. *Pseudomonas* counts were analyzed and determined to be similar to APC. The *Pseudomonas* populations were within a 0.5 CFU/g of the APC. *Pseudomonas* counts from day 2 at 7°C were 3.9 log CFU/g compared to 3.8 log CFU/g for APC. After 12 days storage, *Pseudomonas* counts were 7.3 log CFU/g and APC were 7.5 log CFU/g. The microbial data indicate that APC may be used as an indicator for *Pseudomonas* growth on broccoli. This study revealed that temperature has a greater effect on maintaining microbial growth than wash treatments.

PG activity was measured in broccoli stored at 7°C and 13°C for a 16-d storage period. Measurements were taken to determine if different storage temperatures and wash treatments had an effect on enzyme levels (Table 4). The only significant differences in enzyme activity was for unwashed broccoli stored at 13°C for 12 or 16 days. PG in broccoli stored at 7°C was 5.6 and 6.8 μmol/min/100g compared to PG of 8.9 and 9.9 μmol/min/100g in broccoli stored at 13°C for 12 and 16 days respectively. The increase on days 12 and 16 would indicate the increase due to PG enzymes endogenous to the broccoli. Since microorganisms are removed during the filtration step the enzyme extraction process, enzymes produced by microorganisms would not have an effect on the enzyme activities found in Table 5. When comparing the APC counts from Table 3 to the enzyme activities in Table 5, it can be seen that as APC increased during

Table 4-Analysis of variance of endogenous polygalacturonase activity of washed and unwashed broccoli stored at 7 and 13°C for 16-d.

Source	DF	Enzyme activity mean square	Enzyme activity Pr>F
Day	5	30.54	0.0001
Treatment	1	35.27	0.0001
Temperature	1	9.74	0.0121
Replication	1	0.35	0.6308
Day*Treatment	5	5.22	0.0057
Day*Temperature	5	8.36	0.0001
Treatment*Temperature	1	2.90	0.1669
Error	117	1.50	

Table 5-Endogenous polygalacturonase activity (μmol/min/100g) in washed and unwashed broccoli during 16-d storage at 7 and 13°C.

Storage time (days)	Polygalacturonase activity			
	7°C		13°C	
	Washed	Unwashed	Washed	Unwashed
2	6.62±0.44 def	7.20±1.36 cd	6.37±0.78 defg	7.18±1.06 cde
4	5.90±1.29 efgh	7.08±1.05 cde	5.97±0.90defgh	8.24±2.00 bc
6	5.06±1.15 hij	4.76±0.87 hij	4.49±1.12 ij	5.26±0.43 ghij
8	4.30±0.60 jk	5.98±1.24 defgh	4.40±0.85 ijk	3.14±1.33 k
12	4.80±0.90 hij	5.62±0.82 fghi	4.57±0.81 ij	8.89±1.69 ab
16	5.94±1.65 defgh	6.80±0.34 def		9.87±1.37 a

N=6

Means followed by like letters are not statistically different (P>0.05).

Storage whereas the enzyme activities remained relatively constant. This comparison indicates that there is little or no influence from microbial enzymes on activities shown in Table 5, and suggests that the increase in activity on day 16 was due to endogenous PG enzymes becoming active at 13°C.

Study 2 - *Endogenous enzyme analysis, texture analysis and total enzyme analysis*

Based on study 1, broccoli was stored at 13°C to obtain rapid microbial growth on the broccoli. Two different groups of broccoli were used in this study. One group of broccoli was used for the study of endogenous PG activity using the Gross procedure (1982), and the second group was used to determine a method for measuring endogenous PG activity and PG activity produced by microorganisms together to give a total activity. APC were used to monitor growth of spoilage microorganisms for both endogenous and total activity. There is a significant difference in APC over the 8-d storage period (Tables 6 and 7). The counts on both sets of broccoli were similar, according to Tables 8 and 9. These results are consistent with those of study 1. In studies by Albrecht et al. (1995), Brackett (1989), and Faridaii et al. (1995) the initial APC for broccoli ranged from 5.24 log CFU/g to 6.63 log CFU/g. Differences in counts are to be expected because handling conditions during sampling will vary.

APC in filtrate collected during endogenous PG enzyme extraction were similar to those reported in Table 8. As shown in Table 8, the APC for day 0 was 4.19 log CFU/g. The filtrate count for day 0 was 4.87 log CFU/g. The APC for day 8 was 7.11 log CFU/g (Table 8) and for the filtrate 8.91 log CFU/g. The slight differences in the counts are caused by the dilution factor used to calculate the filtrate APC.

Table 6-Analysis of variance of aerobic plate counts (APC) for broccoli used in endogenous PG analysis.

Source	DF	APC mean square	APC Pr>F
Day	4	5.48	0.0001
Replication	2	1.15	0.0002
Error	17	0.08	

Table 7-Analysis of variance of aerobic plate counts (APC) for broccoli used in total PG analysis

Source	DF	APC mean square	APC Pr>F
Day	4	4.82	0.0001
Replication	2	0.20	0.1311
Error	20	0.09	

Table 8-LSmeans of aerobic plate counts (APC) for broccoli used for endogenous enzyme analysis stored at 13°C for 8-d.

Day	LSmean APC (CFU/g)
0	4.19 d
2	4.57 d
4	5.88 c
6	6.39 b
8	7.10 a

APC incubated at 32°C for 48 h.
N=3
Means followed by like letters are not statistically different (P>0.05)

Table 9-LSmeans of aerobic plate count (APC) for broccoli used for total enzyme analysis stored at 13°C for 8-d.

Day	LSmean APC (CFU/g)
0	4.69 d
2	5.34 c
4	6.04 b
6	6.95 a
8	6.97 a

APC incubated at 32°C for 48 h.
N=3
Means followed by like letters are not statistically different (P>0.05)

PG activity varied with days of storage (Table 10), but the measured activities did not significantly increase over the 8-d storage period ($p<0.05$)(Table 11). The enzyme activities determined with the Gross method are true PG endogenous activities because the microorganisms are lost during filtration in the extraction procedure. As shown, the day 8 level of 4.25 μmoles/min/100g is lower than days 2 through 6 at 6.1 to 7.9 μmoles/min/100. Possibly these day-to-day fluctuations could be attributed to variation in plant material, and to the day-to-day extraction procedure. Unlike the studies by Jen and Robinson (1984) and Tucker et al. (1980), there was no consistent increase in PG activity in broccoli stored for 8-d at 7°C.

Texture analysis was performed on broccoli cores, 2 cm in length, taken from stalks stored in the same bags of broccoli used for endogenous PG measurement. This was done to keep storage conditions constant. The stalks were cored on the same day as the PG measurement in order to eliminate microbial influence on the cores. There was no significant change in texture of broccoli cores stored over the 8-d period (Tables 11 and 12). There is no consistent trend in the curve due to the plant to plant variation. As shown in Table 12, there is no significant decrease in tissue softening from day to day over the 8-d storage period. The lack of softening would relate to the levels of endogenous PG found in Table 9. The absence of softening and the low levels of PG would indicate that endogenous PG does not have a significant effect on tissue softening of broccoli, at this storage temperature and time.

As previously discussed, in this study that microorganisms were removed from the broccoli tissue in the filtration step for the enzyme extraction process described by Gross (1982). The removal of the microorganisms gives an endogenous measurement for

Table 10-Analysis of variance of endogenous polygalacturonase activity for broccoli stored at 13°C over 8-d storage period.

Source	DF	PG level mean square	Pr>F
Day	4	21.89	0.0001
Replication	2	1.49	0.5766
Error	36	2.66	

Table 11-Endogenous polygalacturonase (PG) activity of broccoli stored at 13°C for 8-d storage period.

Day	PG activity (μmol/min/100g)
0	4.13±1.49 b
2	6.52±1.34 a
4	7.93±1.80 a
6	6.08±1.31 a
8	4.25±2.00 b

N=3
Means followed by like letters are not statistically different (P>0.05)

Table 12-Analysis of variance of maximum force required to penetrate cores from the center of broccoli stalks stored at 13°C for 8-d storage period.

Source	DF	Kg force (millions) mean square	Pr>F
Day	4	24.7	0.4206
Replication	2	21.6	0.6512
Error	64	25.0	

Table 13-Lsmeans of maximum force required to penetrate cores from the center of broccoli stalks stored at 13°C for 8-d storage period.

Day	Lsmean (kg force/millions)
0	6690.40 a
2	6593.36 a
4	7437.57 a
6	7526.00 a
8	6907.29 a

Means within columns followed by like letters are not statistically different (P>0.05)

the level of PG activity in the plant tissue. Literature searches revealed no procedure for measuring total PG activity of endogenous PG enzymes plus PG enzymes produced by spoilage microorganisms. Total activity was measured by modifying the Gross extraction procedure. The initial filtration step was deleted to prevent the removal of microorganisms. Everything else in the procedure remained the same. Unpurified samples taken from the filtrate collected were assayed. The absorbance readings produced from these assays were very high and unreadable. The purification step is important in order to remove debris and interfering compounds, such as chlorophyll, that cause very high absorbance readings at 276 nm. The analysis of total enzyme activity is reported in Table 14. There was significant increase in enzyme activities during storage. The total PG activities shown in Table 15 are higher than the endogenous activities in Table 9. The extra amount of PG is due to PG produced by microorganisms. The enzyme activities (Table 15) fluctuated during first 6 d of storage, similar to endogenous enzymes (Table 10). This fluctuation could be due to variation in plant material and bacterial levels. No conclusion could be drawn on how much of the activity level microbial enzymes contributed.

This study indicated that the endogenous enzyme content of minimally processed broccoli did not increase during storage at 13°C over an 8-d storage period. The total activities observed were higher than the endogenous levels. Because interfering compounds were eliminated by purification, the increased PG enzyme levels used for total activity could be a result of the production of PG produced from spoilage microorganisms.

Table 14-Analysis of variance of total polygalacturonase activity for broccoli stored at 13°C over 8-d storage period.

Source	DF	PG level mean square	Pr>F
Day	4	52.30	0.0016
Replication	2	53.45	0.0076
Error	33	9.37	

Table 15-Total polygalacturonase (PG) activity produced by spoilage microorganisms And endogenous PG of broccoli stored at 13°C for an 8-d storage period.

Day	PG activity (μmol/min/100g)
0	12.87±6.27 b
2	16.54±1.87 b
4	15.72±2.12 b
6	16.97±3.32 b
8	20.60±2.98 a

N=3; Means followed by like letters are not statistically different (P>0.05)

Study 3 - *Enzymes produced by spoilage microorganisms*

Sterile homogenized broccoli broth was inoculated with spoilage microorganisms (approximately 3 log CFU/g) obtained from spoiled broccoli. Preliminary experiments conducted using C-F-C agar specific for the isolation of *Pseudomonas* spp. indicated that broccoli initially contained approximately 3 log CFU/g *Pseudomonas* spp. The inoculum was a mixed culture of *Pseudomonas* and other spoilage microorganisms that have been closely associated with the spoilage of broccoli and other minimally processed vegetables. According to the study by Wimalajeewa et al. (1987), *Pseudomonas fluorescens* was considered to be the predominant microorganism associated with broccoli spoilage. Hildebrand (1989) and Nguyen-the (1989) support Wimalajeewa et al. (1987) results but concludes that a specific strain of *fluorescens* called *Pseudomonas marginalis* has the strongest effect on broccoli spoilage. Studies conducted by Brocklehurst et al. (1987), Nguyen-the and Carlin (1994), and Robbs et al. (1996) found that *Erwinia carotovora*, *Erwinia herbicola*, and *Xanthomonas* spp. are closely related to spoilage of minimally processed vegetables. In this study, the APC changed significantly during storage (Table 16). The APC (Table 17) increased approximately 2.5 log from day 1 to day 2. The rapid microbial growth rate may be due to the abundance of nutrients that were available in the sterile homogenized broccoli broth. The abusive growth temperature of 13°C also provided a desirable environment for the organisms to grow.

Extracellular PG samples were extracted using a modified procedure of Hao and Brackett (1994). The measurements taken could not be used for statistical analysis due to their inconsistent and unreliable absorbance readings. The high absorbance readings obtained at 276 nm when measuring the microbial enzyme activity are due to the

Table 16-Analysis of variance of aerobic plate counts (APC) for broccoli used in Microbial produce polygalacturonase analysis

Source	DF	APC mean square	APC Pr>F
Day	3	9.54	0.0001
Replication	2	0.23	0.0664
Error	14	0.07	

Table 17-Microbial LSmeans of aerobic plate counts (APC) for analysis of enzymes produced by spoilage microorganisms grown in broccoli media at 13°C for a 4-d storage period.

Day	LSmeans APC
1	4.88 c
2	7.20 b
3	7.20 b
4	7.77 a

APC incubated at 32°C for 48 h.
N=3
Means within columns followed by like letters are not statistically different (P>0.05)

presence of interfering compounds in the broccoli medium extracts. Similar results due to interfering compounds were found by Jen and Robinson (1984). The addition of the desalting step for this study could add to the clean up and purification of the extracts which would result in more reliable results.

Broccoli cores taken from the stalk were cut to 2 cm in length. The cores were placed in a sterile broccoli medium and used as controls. Sample cores were placed in broccoli medium inoculated with spoilage microorganisms. The procedures were conducted in order to determine the effect of pectinolytic enzymes produced by spoilage microorganisms on tissue softening of broccoli. As shown in Table 18, there are no significant differences between the control samples and the microbial treated samples for days 0, 1, 2, and 3. Table 18 shows a significant difference between the control sample and treated sample on day 4. A direct comparison of the control samples that were matched to the core samples stored in the inoculated medium indicated that there was a 2270 g decrease in the firmness of the treated sample from the control. Table 18 also shows a decrease in firmness on day 5, although not as dramatic as day 4. The softening on day 4 for can be related to the microbial counts shown in Table 17. The counts had reached peak level which suggests enzyme production was taking place and starting to take effect.

Table 18-LSmeans for the maximum force (g) obtained during compression of broccoli cores stored at 13°C over a 5-d storage period.

	Lsmean Maximum Force (g)	
Day	Control	Microbial
0	4636.75 a	4722.50 a
1	4278.90 ab	4368.70 ab
2	4317.22 ab	4042.82 ab
3	4207.50 ab	3924.50 ab
4	4591.83 a	2324.33 c
5	3222.99 bc	2431.99 c

N=6; Means within columns followed by like letters are not statistically different

Chapter 5

Summary and Conclusions

From the data in study 1, day to day washing with 200 ppm chlorine did not have a significant effect on preventing microbial growth on minimally processed broccoli during 16-d storage at 7 and 13°C. However, washed broccoli maintained lower microbial populations at 7°C than unwashed broccoli for up to 12-d. The data did indicate that there was an initial reduction of up to 2 log CFU/g when the broccoli was washed at day 0. Data from storage of broccoli for 16-d at an abusive temperature of 13°C showed that microbial growth was accelerated, and reached a stationary phase at day 8. The microbial growth rate at 7°C was slower. C-F-C counts for *Pseudomonas* were similar to APC over 16-d. Endogenous PG activity remained constant during the first 12 days of storage. After 12 and 16 days there was an increase in PG activity from the broccoli stored at 13°C compared to the broccoli stored at 7°C.

Results from study 2 confirm that endogenous PG activity in broccoli does not increase during storage. There was no significant change in texture of broccoli stems stored at 13°C for 8-d. The enzyme and texture data indicated that endogenous PG activity does not have a significant effect on texture of broccoli stored at 13°C for an 8-d storage period. APC performed on filtrate collected during enzyme extraction from broccoli tissue were related to the surface APC found on broccoli. Therefore, the typical extraction method removes microbial PG leaving only endogenous PG from the broccoli. A modified method of extraction was used to eliminate the loss of microorganisms during filtration. The desalting of the enzyme extraction from both the broccoli and

microorganisms eliminated interfering compounds that would be measured at 276 nm. Total PG activity of spoilage microorganisms and endogenous PG, increased significantly, compared to endogenous PG activity. The increase in PG activity could be attributed to the presence of PG enzymes produced by the spoilage microorganisms. Using the two enzyme extraction procedures allows the comparison of PG produced by spoilage microorganisms and by minimally processed vegetables.

PG activity produced by spoilage microorganisms extracted from broccoli medium could not be statistically analyzed due to inconsistent and unreliable results. The presence of interfering compounds from the broccoli broth resulted in high absorbance readings at 276 nm. The addition of the desalting or purification step in the extraction technique could possibly reduce the interference problem. Texture of broccoli cores did not change during the first 3 days of storage. On day 4, there was a significant softening of broccoli cores stored in the inoculated medium compared to cores stored in the sterile medium. Therefore, the decrease in texture could be related to the APC on day 4. The abundance of microorganisms produce high levels of PG which in turn softens the broccoli tissue.

The conclusions of this research are that 1) washing can reduce microbial populations initially but does not maintain the counts at low levels during storage, 2) total enzyme activity can be measured for PG produced by microorganisms and for endogenous by using two separate extraction methods, 3) broccoli texture is more affected over an 8-d storage period at 13°C by PG from spoilage microorganisms than by PG endogenous to broccoli.

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