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Title: Characterization of Factors Affecting Pectinmethylesterase Activity in Cucumber Fruit

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Pectinmethylesterase (PME) activity in fresh cucumber fruit was determined. The effects of several cationic species, temperature, pH, and acetic acid on the activity of cucumber PME was investigated. The efficacy of blanching, infiltration with $CaCl_2$ or acetic acid, and fermentation with 0 and 40 mM $CaCl_2$ on inhibition of cucumber PME activity were evaluated. Over 50% of PME activity was present in the skin and 75% occurred within the outer 3 mm of the fruit. Maximum stimulation of PME occurred at 125 mM NaCl, 200 mM KCl, and 5 mM $CaCl_2$. Higher levels of each cation demonstrated inhibition of PME activity. 50% inhibition was observed at 750 mM NaCl, 800 mM KCl, and 200 mM $CaCl_2$. Optimum pH was 8.0 and acid pH conditions greatly reduced activity. PME exhibited no activity at pH 4.0. PME activity responded typically to temperature variations with maximum activity occurring at 50°C. The temperature coefficient Q_{10} for PME activity

between 10 and 40°C was 1.24. PME activity was slightly stimulated by increased levels of acetic acid and reached its maximum at 1.5% acetic acid at pH 7.5. No inhibitory effect on PME was detected at acetic acid levels of 0 to 2.0%. Inhibition of PME by NaCl and $CaCl_2$ was reversible. High levels of NaCl (1 M) stabilized PME activity while incubation of PME activity in low levels of NaCl (0.24 M) resulted in loss of activity over time. Both high (500 mM) and low (5 mM) levels of $CaCl_2$ imparted stability to PME activity. No detectable PME activity remained in cucumbers after 15 days of fermentation. Addition of $CaCl_2$ (40 mM equilibrated) to the fermentation brine caused a rapid reduction in PME activity during the first 6 to 12 hours after brining. After 24 hours of brining there was no difference in PME activity due to $CaCl_2$ addition. Acetic acid infiltration at high levels (>10%) effectively reduced the pH of skin tissue to near 4 and resulted in complete inhibition of PME activity.

The most effective treatment for controlling cucumber PME activity was rapid pH reduction by acetic acid infiltration and resulting in PME inhibition. Infiltration with very high $CaCl_2$ levels (>500 mM) may also be beneficial toward accomplishing PME inhibition. Rapid inactivation prior to brining or within 6 to 12 hours after brining is necessary to achieve effective control of cucumber PME activity.

CHARACTERIZATION OF FACTORS AFFECTING PECTINMETHYLESTERASE ACTIVITY IN CUCUMBER FRUIT

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INTRODUCTION

Softening of cucumber tissue occurs during brine storage particularly when low levels of NaCl are used (Tang and McFeeters, 1983). Chemical changes that take place in the cell wall and middle lamella have been related to softening of brined cucumber tissues. Employing high concentrations of salt in brine is an ancient practice to preserve fruits and vegetables (Pederson, 1971). The use of Ca^{++} and/or high concentrations of NaCl in fermentation and storage brines have been shown to prevent softening of cucumber pickles (Buescher et al., 1979; Buescher and Hudson, 1984; Hudson and Buescher, 1980). When added initially to cucumber brine, calcium could most effectively maintain cucumber tissue firmness during brine storage (Buescher et al., 1981; Tang and McFeeters, 1983).

Calcium pectates have been known to maintain the structural integrity and firmness of plant tissues (Grant et al., 1973). Formation of calcium pectates have been shown to be responsible for resistance to softening of plant tissues by polygalacturonase (Bateman, 1964). Bartolome and Hoff (1972) have proposed that demethylation of pectins by pectinmethylesterase (PME) contributes to

tissue firming by creating more free carboxyl groups for Ca^{++} binding.

However, recent evidence has indicated that extensive demethylation of cucumber pectins results in a significant loss of texture in cucumber pickles (Hudson and Buescher, 1986). It appears that greater texture can be maintained in the final pickle product by controlling the amount of demethylation of pectins occuring in cucumbers during brining. One of the major changes in the chemical composition of cucumber cell walls during brining is rapid and extensive demethylation of pectins (Hudson and Buescher, 1986; McFeeters et al., 1985; Tang and McFeeters, 1983). Pectic substances in fresh cucumbers contain from 35-50% free carboxyl groups suggesting that there are abundant sites for Ca^{++} binding without the need for additional demethylation (Fukushima, 1978; Hudson and Buescher, 1985).

Michel and co-workers (1982) have investigated the relationship between degree of esterification and the configuration of pectin macromolecules. They proposed that stiffness of the pectin macromolecule increases with increasing pectin methoxyl content. As pectin demethylation proceeds, a transition from a rigid molecular structure to a relaxed elementary fibril configuration takes place (Leeper and Dull, 1972).

Previous studies have shown that Ca^{++} protected against tissue softening by reducing the solubilization and

deesterification of pectic substances indicating that firm tissues are associated with the formation of insoluble and methylated pectins (Hudson and Buescher, 1985; 1986). Furthermore, these authors determined that methods which protect against excessive demethylation of pectins appear to be important in retarding softening of cucumber pickle tissue during storage in brine (Hudson and Buescher, 1986).

Since cucumbers contain significant levels of PME (Bell et al., 1951) it is likely that demethylation is an enzymatic process in cucumber tissue. Therefore, methods of controlling the activity of PME in fresh and brined cucumbers could result in maintenance of greater levels of methylated pectins and, consequently, enhanced textural quality of cucumber pickles. Additional research is needed to determine treatment methods which are effective in controlling PME activity in cucumbers.

PME has been shown to be activated by salts. Purified tomato PME activity was highest in the presence of either 0.005 M $CaCl_2$ or 0.05 M NaCl (Lee and Macmillan, 1968). Maximum PME activity was detected near pH neutrality for most plants (Jansen et al., 1960; Reed, 1966). Optimum PME activity of purified tomato was observed at pH 8.5 (Lee and Macmillan, 1968), but a lower optimum has been found for apple PME (pH 6.6) (Pollard and Kieser, 1959). Plant PME, including the enzyme in cucumber tissue (Bell et al., 1951), is generally rather heat stable (McFeeters et al., 1985). Tomato PME showed 50% inactivation after 1 hour at

70°C in 0.1 M NaCl and at pH 6 (Reed, 1966). McFeeters et al. (1985) found that an 81°C blanch caused complete cucumber PME inactivation, but up to 20% reactivation occurred during storage.

The first objective of this investigation was to study the effects of several ionic species, temperature, and pH on the activity of cucumber PME. These factors were studied in order to develop methods of controlling the activity of this enzyme in cucumber fruit. Therefore, the second objective was to employ those factors most effective in controlling PME activity in fresh and brined cucumbers.

Two seperate experiments were conducted. In experiment I the effects of NaCl, KCl, $CaCl_2$, acetic acid, pH and temperature on cucumber PME activity were determined. Experiment II was designed to evaluate the efficacy of blanching, infiltration with $CaCl_2$ or acetic acid, and fermentation with 0 and 40 mM $CaCl_2$ on inhibition of cucumber PME activity.

LITERATURE REVIEW

The "art" of making pickles predates recorded history. The commercial brine preservation of cucumbers (Cucumis sativus L.) and other vegetables is essentially the same as it has been since man first learned to store certain foods by the use of salt (Pederson, 1971). Cucumber pickles are manufactured either directly from fresh cucumbers (fresh pack) or from cucumbers that have been fermented in salt brine (cured pickles). Today, approximately 40% of the United States pickling cucumber crop is temporarily preserved by brine fermentation and storage (Fleming et al., 1987).

The commercial brining operation during the harvest season can be described as placing cucumbers in 2,000- to 20,000- gallon tanks along with a brine solution in which the cucumbers ferment (Fleming et al., 1987). The tanks, after being filled with cucumbers, are headed with false heads. False heads are wooden headboards that are positioned to restrain the cucumbers below the brine surface (Bell et al., 1950). Dry salt is added on the false head of the tank to maintain a salt level of 12-16%, which otherwise would become diluted by the water from the cucumbers (Etchells et al., 1951). The open top tanks are stored outdoors to permit exposure of the brine surface to sunlight. Sunlight retards growth of film yeasts and other oxidative microorganisms on the brine surface (Frazier and

Westhoff, 1978). The initial salt level ranges from about 5-8% during fermentation to permit rapid growth of lactic acid bacteria (Bell and Etchells, 1961). Under these conditions an active acid fermentation resulting from the growth of salt-tolerant, lactic acid bacteria begins within a day or so after the cucumbers are brined. Lactic acid bacteria convert fermentable sugars to lactic acid and other end products. Glucose and fructose are two major fermentable sugars in cucumber (McCombs et al., 1976). Glucose is fermented by lactic acid bacteria to lactic acid, ethanol, and carbon dioxide. The end products of fructose fermentation are lactic acid, acetic acid, carbon dioxide, and mannitol (Gottschalk, 1979). The traditional fermentation of cucumber takes 6 to 9 weeks for completion (Frazier and Westhoff, 1978). In most instances the salt concentration is gradually increased after fermentation by adding more dry salt so that a concentration of about 12-16% NaCl is obtained after about six weeks (Fleming et al., 1987). The high salt level serves as a preservative against spoilage, particularly softening during storage, and to lower the freezing point of the brine. Brine stock cucumbers are removed from the tanks for further processing during the year as needed (Fleming et al., 1987). For processing, the pickles are removed from the storage brine (spent brine), desalted by holding and washing in several volumes of fresh water, and then packed in vinegar, salt and various flavors and/or color. The final products

contain the desired salt concentration of the specific product being manufactured. Salt concentration is 2-4% for certain types of dill pickles (Fleming et al., 1987).

Disposal of large quantities of spent brine and brine generated during desalting is a major problem confronting the pickling industry (Hudson and Buescher, 1980; Hudson and Buescher, 1985; McFeeters et al., 1978). Several methods have been explored for re-using the spent brine or for reducing the levels of spent brine (Palnitkar and McFeeters, 1975; Stevenson et al., 1979). Many of these methods are expensive and others are still in the developmental stages.

During fermentation and storage of cucumbers the industry is often faced with problems of spoilage known as "softening" (Bell et al., 1950; 1955; 1961; Etchells et al., 1958). Cucumbers may become mushy and lose their firmness completely or develop localized softening which is less severe. The economic loss to the industry is considerable when soft cucumbers must be used in lower quality products. In order to insure that acceptable texture is maintained in finished cucumber pickle products, the factors which may influence texture during fermentation, brine storage, processing and distribution must be controlled. One of the factors that influences texture is the chemistry and structure of the cell wall (Van Buren, 1979). Therefore, the chemical changes that occur in the cell wall and middle lamella components of

fruits and vegetables must be controlled in order to control factors which may influence texture.

The primary cell wall is the predominant structural component in edible plants. Secondary cell walls are almost absent from mature fruits and their presence in vegetables usually makes them too tough and fibrous (Nelmes and Preston, 1968).

Plant cell walls are composed of cellulose fibrils imbedded in a matrix consisting largely of pectic substances, hemicelluloses, water and smaller amounts of proteins, lignins, and lower molecular weight solutes (Albersheim 1974; Preston 1974). Covalent, hydrogen, and ionic bonding are believed to be involved in the bonding among the polymeric cell wall and middle lamella components (Albersheim, 1974). Oakenfull and Scott (1984) have shown that the network of polysaccharide molecules in gels of high methoxyl pectins is stabilized by a combination of hydrophobic interactions and hydrogen bonds. Crystalline structure is important in the bonding of cellulose microfibrils (Goodwin and Mercer, 1982). Northcote (1958) has indicated this composition in his classic model, shown in Fig.1.

A major advance in dealing with the complexity of the cell wall was the model proposed by Keegstra et al. (1973) to describe the covalent interactions of pectin, cellulose, hemicellulose and protein. The model (Fig. 2) has been subjected to periodic review and updating by

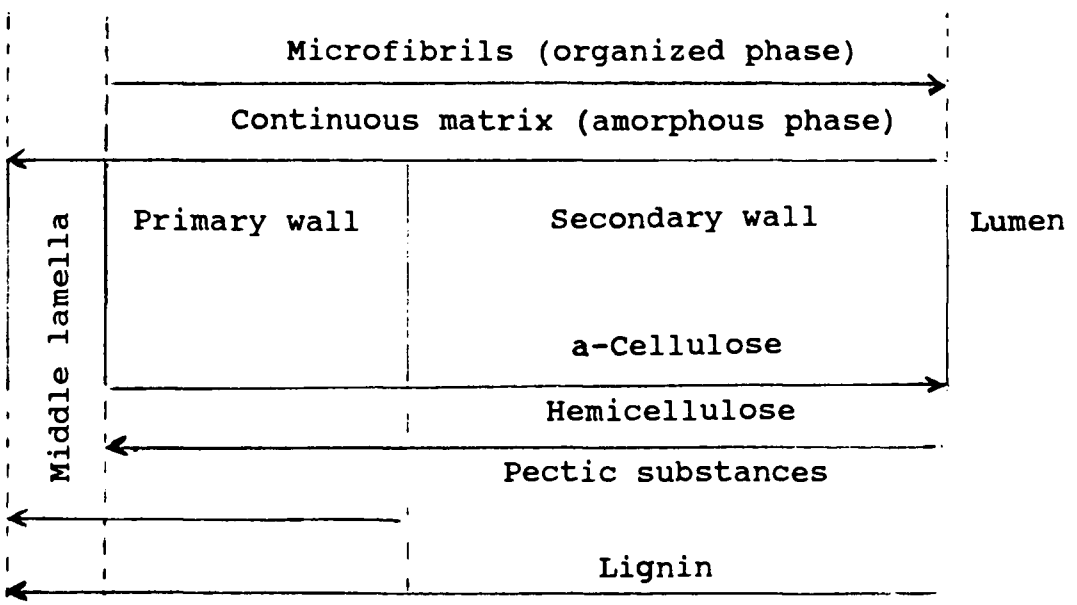

Fig.1 Variation in the concentration of the major components of plant cell walls (Northcote, 1958).

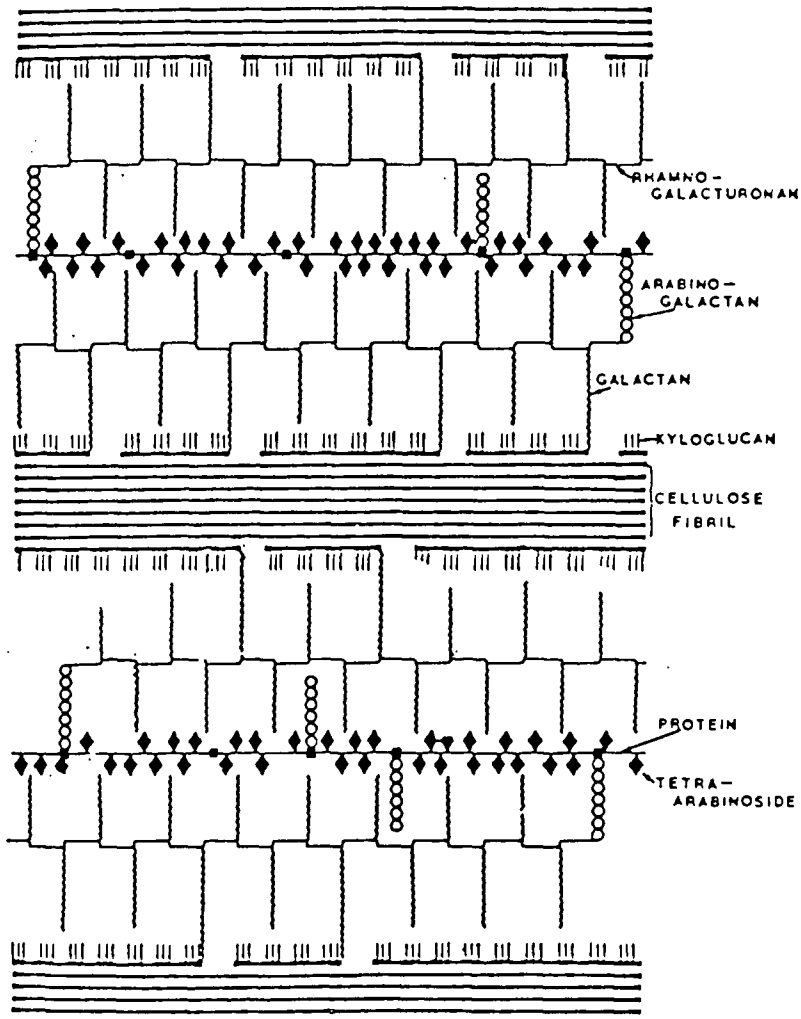

Fig.2 Tentative structure of sycamore cell walls (from Albersheim, 1976).

several investigators (Albersheim, 1976; Darvill et al., 1978). In the initial network proposed, pectic substances consisted of an a(1-4) linked polygalacturonan mainchain, linked every 14 or so residues by two anhydrorhamnose units (Rees, 1969). This main chain is crosslinked by arabinan and galactan chains either seperately or as an arabinogalactan, to hemicellulose (Northcote, 1972). These neutral sugars were attached to the mainchain at the anhydrorhamnose residues. The sidechains were believed attached covalently at their other extremity to the xyloglucans of hemicellulose. Hemicellulose in turn was believed hydrogen bonded to cellulose (O'Beirne, 1980).

The middle lamella may be considered an extension of the matrix material of the primary cell wall and since it is the outermost portion of the plant cell, it plays the primary role in interacellular adhesion (Van Buren, 1979). Pectic substances constitute a major portion of the middle lamella (Van Buren, 1979). They contribute to mechanical strength of the wall and to the adhesion between cells (Doesburg, 1965). Pectic substances are more soluble in water than other cell wall polymers and are more chemically reactive (Hobson, 1981). Several factors, such as ripening, endogeneous and exogeneous enzymes, storage, and cooking contribute to textural degradation of fruits and vegetables (Doesburg, 1965). Softening is accompanied by significant changes in the characteristics of the pectic substances

(Deuel and Stutz, 1958; Hulme, 1970; Hobson, 1981; Pressey, 1977).

The polygalacturonan main chains of pectic substances isolated from plant cell walls possess carboxyl groups on the galacturonic acid residues which are extensively esterified with methyl alcohol (Doesburg, 1965). The kinks caused by the rhamnose inserts, neutral sugar side chains and the molecular charge contributed by the carboxyl groups all affect the gelling properties of pectic substances.

Rees (1969) developed the concept that carbohydrate gels are formed by adhesion between neighboring chains at "junction zones". Junction zones consist of two adjacent segments of polysaccharide chains varying in length from 18 to about 250 galacturonic acid units, increasing with the degree of methoxylation (Oakenfull and Scott, 1984). In charged molecules of pectic substances with a low degree of esterification (DE) and high pH, it is proposed that calcium binds between the chains at junction zones involving free carboxylic groups in the manner of eggs in an egg box (Rees 1975; Thom et al., 1982). This proposed model explains firming characteristics in plant tissues in presence of calcium. The egg box model demonstrates that in pectic substances where there are long blocks containing free carboxyl groups, pectin polymers line themselves in linear fashion next to one another. It is suggested that the free carboxyl anions bind divalent calcium cations which fit precisely in the voids between these adjacent

chains and form a tight gel matrix (Thom et al., 1982) shown as Figure 3.

Calcium is an effective regulator of vegetable texture and it may be used in several situations to protect against excessive softening (Buescher et al., 1979; 1981; Buescher and Hudson, 1984; Hudson and Buescher, 1980; Fleming et al., 1987). The addition of calcium to fresh products is primarily limited by the difficulty of its incorporation and phytotoxicity, while in processed foods its addition is limited by the creation of adverse taste (Bruce and Callow, 1934; Oberleas et al., 1966). It is within the middle lamella and cell walls that calcium plays an important role in the protecting against excessive softening by reacting with the pectic substances to form calcium pectates (Grant et al., 1973). Calcium pectates are presumed to be responsible for maintaining the structural integrity and firmness of plant tissues (Grant et al., 1973). Formation of calcium pectates has been shown to be responsible for preventing or retarding softening of plant tissues by polygalacturonase (PG) (Bateman, 1964). PG is a depolymerizing enzyme and degrades pectin by hydrolysis of the glycosidic bonds of the main chain of polygalacturonic acid (Deuel and Stutz, 1958; Doesburg, 1965; Loewus, 1973). PG is found in many plant tissues, especially climacteric fruits. Endo-PG is most important in fruit softening and attack by microorganisms.

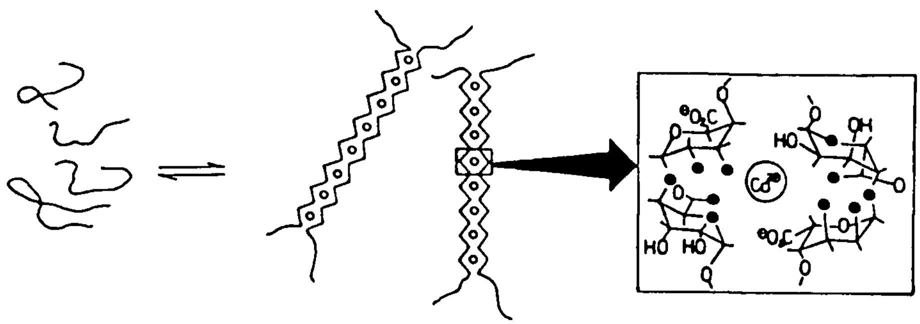

Fig.3 Schematic representation of the "egg-box" model for the sol-gel transition in alginates. The disposition of groups coordinating the divalent cations is also shown andfor clarity the residues have been moved apart (from Thom et al. 1982).

Only one study has detected the presence of endo-PG in the centers of large over-mature cucumbers (McFeeters, 1980). Exo-PG has been detected in mature (over-ripe) fruits by Pressey and Avants, (1975). In addition to plants, PG is also produced by parasitic and saprophytic micro-organisms (Chesson, 1980). Microbial PG, whether introduced into the brining tank by external sources attached to the cucumber fruits, flowers, or blossoms (Etchells et al., 1958) or produced in the brining tank by over-ripe or moldy cucumbers, can greatly reduce the firmness of brined cucumbers.

Calcium is presently being used commercially to protect cucumbers against softening by PG, cellulase and other softening agents during fermentation and brine storage. The use of calcium in fermentation brines makes it possible to reduce NaCl levels in fermentation and storage brines. Therefore the desalting process can be eliminated, substantially reducing the volume of waste water and spent brine (Buescher et al. 1979; Buescher et al. 1981; Hudson and Buescher, 1980). Presence of calcium in brines at the time of adding fresh cucumbers caused enhancement of firmness. On the other hand when calcium was added after cucumbers were brined, little or no protection against softening was observed.

The classical explanation of calcium enhancement of firmness is that calcium intensifies middle lamella-cell wall rigidity by formation of calcium pectate gels (Grant

et al. 1973). Hudson and Buescher (1985) concluded that calcium prevents softening by protecting pectic substances from degradation and perhaps more importantly by retarding excessive demethylation of pectic substances. Calcium appears to have at least two major functions in protecting against tissue softening: (1) forming inter- and intramolecular bonds between demethylated sites (Grant et al., 1973; Van Buren, 1979) and (2) reducing demethylation of pectins (Hudson and Buescher, 1985; 1986). According to Bartolome and Hoff (1972) demethylation of pectins has been proposed as a mechanism for tissue firming by creating more free carboxyl groups for calcium binding. Pectin substances of fresh cucumbers are from 35-50% de-esterified (Fukushima, 1978; Hudson and Buescher, 1985). This should permit enough sites to form calcium pectates without the need for additional free carboxyl groups. Hudson and Buescher (1986) demonstrated that firmness of cucumber mesocarp tissue was directly related to the DE of pectins when the DE was less than 12.3 (Fig. 4). They indicated that when the DE values were greater than 12.3, tissue firmness was unaffected by the level of methylated pectins. Extensive demethylation of pectins occurred rapidly during brining with most changes taking place within two weeks (Hudson and Buescher, 1985; Tang and McFeeters, 1983).

Dull and Leeper (1975) and Leeper and Dull (1972) have demonstrated that demethylation of pectin results in a

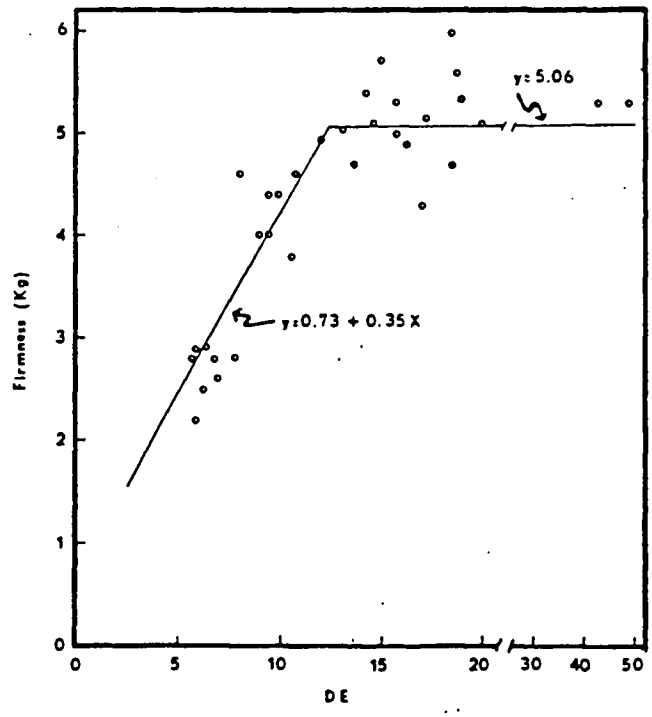

Fig.4 Relationship between firmness of cucumber mesocarp tissue and the degree of esterification (DE) of pectins (from Hudson and Buescher, 1986).

transition from an ordered rigid coil molecular structure to a linear relaxed elementary fibril configration. Base on their findings, conformational changes in the pectin macromolecule take place when DE is from 0-15. Also Michel et al. (1982) have concluded that the stiffness of the pectin macromolecule increases with increasing methoxyl content. Although these relationships between pectin configuration, DE and rigidity were based on observations of purified pectins, they could provide a basis for explaining the relationship found between firmness of cucumber tissues and the DE of pectins.

The enzyme responsible for demethylation of pectic substances is pectinmethylesterase (PME) (Chesson, 1980). It is also known as pectyl-hydrolase, pectinesterase (PE), pectase and pectin methoxylase (Doesburg, 1965). PME catalyzes the hydrolysis of methyl esters that are adjacent to free carboxyl groups of D-galacturonate and is highly specific for D-galacturonate (Doesburg, 1965). PME is found in all species of higher plants and in many bacteria and fungi (Reed, 1966). Deesterification by PME from plants proceeds linearly along the chain, creating blocks of free carboxyl groups (Doesburg, 1965). About half of plant PME activity occurs at the reducing end of the molecule with the remaining activity occurring at some secondary position on the chain, probably near free carboxyl groups (Hobson, 1981). The fungal PME generally has a pH optimum in the acid range and is activated by salts (Reed, 1966). The

optimum salt concentration varies with the pH as follows: at pH 3.5 optimum activity in the presence of either 0.1 M $CaCl_2$ or 0.5 M NaCl; at pH 5.5 optimum activity in the presence of either 0.01 M $CaCl_2$ or 0.2 M NaCl (Reed, 1966). Bacterial PME has pH optimum on the range of 7.5 to 8.0 (Reed, 1966). The optimum pH for activity of PME from most plants is in the alkaline range (pH 7.5-8.0) (Bell et al., 1951; Pressey and Avants, 1972; Van Buren et al., 1962). Cucumber PME is activated by 0.15 to 0.20 M NaCl (Bell et al., 1951) or 0.005 M $CaCl_2$ in the case of tomato (Lee and Macmillan, 1968). Optimum temperature varies depending on the source of PME. Optimum temperature for citrus, apple, tomato, and grape are 65, 55, 80, and 40 C, respectively (Chesson, 1980).

The importance of PME, particularly in the presence of low levels of calcium, is to prepare pectin for attack by PG. In plant tissues, demethylation by PME is a precondition for PG hydrolysis of pectic substances since completely methylated pectin can't be attacked by PG (Deuel and Stutz, 1958; Pressey and Avants, 1982).

Leeper and Dull (1972) have demonstrated that demethylation of the pectin macromolecule causes transformation from a rigid to a relaxed molecular structure. Extensive demethylation of cucumber contributes to significant loss of texture in cucumber fruit (Hudson and Buescher, 1985). Cucumber contains significant amounts of PME (Bell et al., 1951) which causes demethylation. PME

activity may have a direct effect in softening of brined cucumber fruit. As a result, the texture of brined cucumbers may be maintained by controlling PME activity.

MATERIALS & METHODS

Source of Cucumbers

Fresh cucumbers (Pioneer variety) were obtained from Steinfeld's Pickle Company and a local wholesaler located in Portland, Oregon. Size no. 3 (3.8-5.1 cm. diameter) fruit, free from decay and mechanical injury, were used after washing with deionized water.

PME Assay

A. Preparation of Solutions

Desalting solution included 0.05 M sodium phosphate buffer (pH 7.5) and 0.01% Thimerosal (preservative) in deionized water.

Extracting solution was made of 1 M NaCl, 1% PVP (MW=360,000), 0.1 M sodium phosphate buffer (pH 7.5), and 0.01% Thimersal which was brought to volume with deionized water.

The substrate consisted of 1.0% citrus pectin (DE=62%) and 0.01% Thimerosal which was brought to 500 ml with deionized water. To prevent any lump formation, the pectin was added slowly in small quantities and the solution was mixed on low heat at all times. This 1% pectin solution was diluted to 0.5% prior to the assay with deionized water. The pH of the substrate was adjusted to 7.5 with 0.1 N NaOH just prior to use and was used within one day. The 1% pectin solution at a pH of 2.9 was stored at $4^{o}C$ for further use.

NaOH (0.01 N) was prepared and standardized with standard potassium hydrogen phthalate by the AOAC method (1980).

B. Extractions and Analysis

Fresh cucumbers were thoroughly cleaned with deionized water, the end pieces (about 1/4 of the fruit) were cut off and discarded. Skin tissue (outer 1 mm in thickness) from 10 cucumbers was used as the source of PME in all experiments except as noted. A modified method of Luh et al. (1974) was used for extraction of cucumber PME. 100 g of cucumber tissue was homogenized for one minute with 300 ml of extracting solution in a Waring Blendor. To prevent foaming, 2-3 drops of antifoam (octanol) were added. The homogenate was magnetically stirred overnight at 4^{o}C to solubilize PME from cell wall, then filtered through Whatman No. 4 filter paper. The filtrate was centrifuged for 20 min at 10,000xg at 4^{o}C to remove cell wall debris, decanted and desalted through a Bio-Rad P-6DG column (Bio-Rad laboratories, 1984) against 0.2 M NaCl, 0.05 M sodium phosphate buffer, pH 7.5 containing 0.01% Thimersal which was added as a preservative. Bio-Gel P-6DG is a spherical polyacrylamide gel with a molecular weight exclusion limit of approximately 6,000 Daltons which is not subject to enzymatic degradation. 2.5 ml of 0.5% pectin substrate and 100-500 microliter of enzyme were used for determination of PME activity. Both enzyme and substrate were pre-incubated at 30^{o}C for 30 min prior to assay.

PME activity was assayed by a titrimetric method modified from Luh et al. (1974). The free carboxyl groups produced by enzymatic hydrolysis of the methyl ester residues of pectin were continuously titrated with 0.01 N NaOH at constant pH (7.5) using an automatic titrator module (Metrohm pH stat). Instrument controls were set as follows: Dosimat on mode 1, control knob on 2; Impulsomat: EP clock on 1, delay on infinity, dynamics on 1. Initial rates were determined from the slope of the total titrant volume delivered versus time. The standard assay for PME activity was performed at 30°C maintained by a water jacketed reaction vessel. Humidified N_2 gas was maintained in the headspace of the reaction vessel to prevent uptake of CO_2 from the air. A constant pH of 7.5 was maintained in the reaction solutions by titration with 0.01 N NaOH. PME activity was calculated as micromoles of NaOH/minute/gram (fresh weight) of tissue as determined from a 5 min linear titration. PME activity data are presented in international units of micromoles of free carboxyl groups produced per minute. In all experiments triplicates of each treatment were analyzed.

EXPERIMENT I

Localization of PME activity in cucumber fruit

To determine the PME activity in different parts of the cucumber fruit, cucumbers were dissected into five sections: (1) skin (outer 1 mm in thickness), (2) outer-

mesocarp (the 2 mm thick flesh next to skin), (3) inner-mesocarp (the tissue between outer-mesocarp and endocarp), (4) endocarp (locular tissues including the seeds and placenta) and (5) whole fruit without end pieces as shown in Figure 5. The proportion of each section was determined as the percentage of the total fruit weight (fresh weight basis). 50 g of each dissected section was weighed, extracted, desalted, and assayed for PME activity under standard conditions. To determine the weight of each section as a percentage of a whole cucumber, 10 size 3B cucumbers were throughly washed with deionized water and the end pieces were cut off and eliminated. The average weight of a cucumber (without end pieces) was 50.33 g. The cucumbers were then dissected into skin, outer-mesocarp, inner-mesocarp, and endocarp tissues. The dissected sections were weighed separately and recorded as a percentage of a whole cucumber.

Effect of NaCl, KCl, and $CaCl_2$ concentrations on PME activity

A. NaCl Concentration

The effect of NaCl concentration (6.1, 50, 100, 125, 150, 175, 200, 300, 400, 500, 750, 1000 and 1500 mM) on cucumber PME activity was determined. PME from cucumber skin was extracted and desalted. NaCl content of the desalted PME extract was determined using a Cl^- titration method. The amount of Cl^- was measured by titrating a 1.0 ml sample of desalted enzyme in 10 ml deionized water, 4

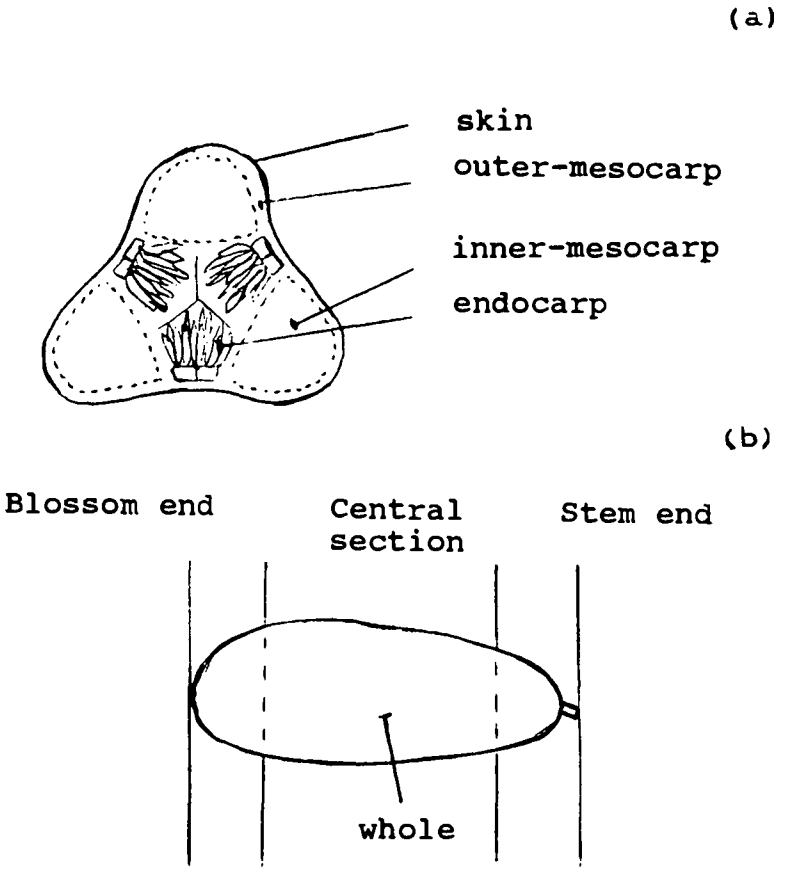

Fig.5 Areas of the cucumber fruit dissected for determination of PME activity. Cross-sectional (a) and longitudinal (b) views of the cucumber are illustrated. Cucumber sections are defined as following: skin (outer 1 mm); outer-mesocarp (2 mm next to skin); inner-mesocarp (the space between outer-mesocarp and endocarp); endocarp (locular tissues including the seeds and placenta); whole fruit without end pieces.

drops 2/3 N H_2SO_4 and 2 drops diphenylcarbazone indicator (5 mg/2 ml methanol) with 0.1 N $Hg(NO_3)_2$ (modified from Sigma Technical Bulletin NO. 830, Sigma Chemical Company, St. Louis, Mo). The background level of NaCl present in the desalted enzyme as well as the Na^+ contributed from the pH adjustment of the substrate to 7.5 with NaOH were taken into account in preparation of the substrate solutions. 0.5% pectin substrate solutions were prepared by diluting 25.00 ml of 1.0% pectin with the following volumes of 3.58 M NaCl: 0.00 ml, 0.72 ml, 1.56 ml, 1.98 ml, 2.40 ml, 2.82 ml, 3.24 ml, 4.91 ml, 6.58 ml, 8.26 ml, 12.44 ml, 16.63 ml and 25.00 ml.

B. KCl Concentration

The effect of KCl concentration (0.05, 50, 100, 150, 175, 200, 300, 400, 500, 750, 1000 and 2000 mM) was determined on cucumber PME activity.

The amount of K^+ present in PME extracts after desalting was measured using ICP (Inductively coupled plasma) spectrometer model 3580 (Boumans, 1987) located at the Environmental Protection Agency laboratory, Corvallis, Oregon. Solutions were diluted by distilled water. Distilled water was prepared by initially passing the water through tin distillation apparatus follwed by charcoal filter and cation-anion (mixed bed) exchange resin. This water was finally redistilled in all glass pyrex. The background level of K^+ was accounted for in preparation of the substrate solutions. Pectin solutions (0.5%) were

prepared by diluting 25.00 ml of 1.0% pectin with the following volumes (ml) of 4.16 M KCl: 0.00, 0.62, 1.25, 1.87, 2.19, 2.50, 3.75, 5.00, 6.25, 9.37, 12.50, and 25.00.

C. $CaCl_2$ Concentration

The effect of $CaCl_2$ concentration ($3.1x10^{-3}$, 1, 2.5, 3.3, 4.2, 5.0, 6.5, 8.3, 10, 20, 30, 40, 50, 75, 100, 250 and 500 mM) was determined on cucumber PME activity. The background level of Ca^{++} in the desalted enzyme was determined as described for K^+ and this level was accounted for in preparation of the substrate solutions. Solutions of 0.5% pectin were prepared by diluting 25.00 ml of 1.0% pectin with the following volumes (ml) of 1.04 M $CaCl_2$: 0.00, 0.05, 0.13, 0.17, 0.21, 0.25, 0.33, 0.42, 0.50, 1.00, 1.50, 2.00, 2.50, 3.75, 5.00, 12.50, and 25.00.

Calcium has a strong affinity to bind with pectic substances and form calcium-pectate gels. Although theoretically hydrogen and hydroxide ions diffuse through a gel and should give an accurate pH stat measurement, gel formation, phase separation, and calcium-pectate precipitation on glass electrodes was minimized by using a pectin concentration of 0.5%, high-speed agitation of the reaction solutions, and a dilute acid rinse of the electrodes after each run using 0.1 N HCl (Beckman, 1983).

The substrates for the NaCl, KCl, and $CaCl_2$ studies were brought up to a final volume of 50.00 ml using deionized water. PME activity was assayed under standard

conditions using 100 microliters of enzyme and 2.5 ml substrate.

Effect of pH on PME activity

PME was assayed at pH values of 4 to 11 at increments of 0.5. PME was pre-incubated at each pH for 30 minutes. Measurements of PME activity above pH of 7.5 is subject to error because pectin undergoes alkaline deesterification in addition to enzymatic deesterification (Lee and Macmillan, 1968). Rates for alkaline deesterification were determined at each pH level with inactivated PME (boiled for 5 min) and corrections were applied to the rates obtained enzymatically by subtracting alkaline from total deesterification.

Effect of temperature on PME activity

After desalting the crude extracts of PME, they were brought to desired temperatures of 2 and 10 to 80^{o}C (increments of 10) for 30 min in closed containers. The enzyme was then assayed under standard conditions.

Effect of acetic acid on PME activity

To test the effect of acetic acid on PME activity, 0.5% pectin was prepared with 0, 0.25, 0.50, 0.75, 1.00, 1.50, and 2.00% acetic acid. The pH of the solutions was buffered to pH 7.5 using dilute NaOH. The NaCl level was kept constant at 363 mM throughout this experiment. The enzyme was assayed under standard conditions.

Reversibility of PME inhibition by NaCl and $CaCl_2$

This experiment was designed to determine if the inhibition of PME by NaCl and $CaCl_2$ was reversible after the removal of these salts by passing through a desalting column. PME crude extract was desalted and the background levels of NaCl and $CaCl_2$ after desalting were determined using atomic absorption spectrophotometry. Atomic absorption spectrophotometry standard conditions for Na^+ assay included preparing stock standard solution of 1000 ppm Na^+ (using NaCl). To overcome interferences of Na^+ ionization, 1500 ppm Li^+ (LiCl) was added to standards and samples. Standard curve was drawn by diluting stock standard solution to up to 1 ppm Na+ (linear range of standard curve). Instrument (Perkin-Elmer model 303) was operated at following settings: wavelength 590 nm (VIS); slit setting of 4; hollow cathode light source lamp; and air-acetylene oxidizing flame (Perkin-Elmer, 1976). The atomic absorption spectro-photometry linear working range for Ca^{++} is up to approximately 5 ppm in aqueous solution. A stock standard solution of 500 ppm Ca^{++} was prepared using $CaCO_3$. To eliminate interferences with Ca^{++} readings, 5000 ppm La^{3+} (using La_2O_3) was added to both standards and samples. The acid concentration of standards (0.75 N HCl) was matched with that of the samples. Operating parameters for instrument model 303 were: wavelength of 422 nm (VIS); slit setting of 4; hollow cathode lamp light source; and reducing air-acetylene flame (Perkin-Elmer, 1976).

To establish the optimum and inhibitory activities of PME by NaCl, desalted PME was assayed with 0.15 and 1.00 M NaCl, respectively. To test reversibility of NaCl inhibition, desalted PME was adjusted to 1.00 M NaCl and incubated for 24 hours at 30°C. After incubation, the enzyme was desalted and assayed at 0.24 M NaCl (the lowest NaCl level attainable with a single pass through the gel filtration desalting column). To evaluate the effect of incubation time on the enzyme, a sample of PME was also incubated in 0.24 M NaCl for 24 hours and assayed at 0.24 M NaCl. To establish the activity of PME at 0.24 M NaCl without prior incubation, a sample of desalted enzyme was assayed at 0.24 M NaCl without any prior incubation treatment. PME assays were conducted under standard conditions.

Optimum and inhibitory activities of PME were established by assaying desalted enzyme at 5 and 500 mM $CaCl_2$, respectively. Reversibility of $CaCl_2$ inhibition was tested by adjusting the desalted enzyme to 500 mM $CaCl_2$ and incubating for 24 hours at 30°C. The effect of incubation time was tested by incubating the desalted enzyme at 5 mM $CaCl_2$ for 24 hours at 30°C. The incubated enzymes were desalted and assayed at 5 mM $CaCl_2$. PME assays were conducted under standard assay conditions.

EXPERIMENT II

Effect of fermentation with and without $CaCl_2$ on PME activity

Cucumbers were tightly packed into 3.8 liter glass jars. Brine solutions containing 4.4% NaCl and 0.1% K-sorbate were first adjusted to pH 3.0 with acetic acid and then to pH 5.0 with 10 N NaOH as described by Buescher et al. (1981). The containers of cucumbers were filled with 1.5 L brine solutions as described above with 0 or 100 mM $CaCl_2$ which provided a pack-out ratio of 60:40 (w/v) of fruit to brine. NaCl, K-sorbate, and $CaCl_2$ were equilbrated to 1.76%, 0.04% and 0 or 40 mM, respectively. Cucumbers were held submerged in duplicate containers and allowed to undergo natural fermentation. All treatments were held at room temperature (23°C) until evaluated. Cucumbers were sampled at 0, 0.25, 0.50, 1, 2, 4, 7, 11 and 15 days of fermentation. Samples were assayed for PME activity under standard conditions.

Effect of $CaCl_2$ and acetic acid infiltration on cucumber PME activity

In this experiment whole cucumbers were submerged in 500 mM $CaCl_2$ solution for 0.5 min, after which the pressure over the solution was reduced to 150 mm Hg and the partial vacuum was maintained for 4.5 min. After release of the vacuum, the cucumbers remained submerged in solution for 5 min. The fruits were allowed to drain for 5 min upon removal from the solution, and rinsed with distilled water

(once) and then drained (Wills and Tirmazi, 1977). Control fruits were infiltrated with water containing no $CaCl_2$. Infiltrated cucumbers were dissected into skin, outer-mesocarp, inner-mesocarp, endocarp, and whole fruit without ends as described earlier. PME activity of each section was measured using standard assay conditions.

Soluble calcium levels were measured according to the method of Suwwan and Poovoiah (1978). Calcium levels were measured in dissected samples of fresh and $CaCl_2$ infiltrated fruit. One gram of each dissected portion of fruit was blended with 20 ml deionized water in a Biohomogenizer (Viospec Products, model M-133-1281-0). The sample was centrifuged for 15 min at 22,500xg. The supernatant was passed through Whatman No. 541 ashless filter paper and collected in 100 ml volumetric flasks. The pellet was washed two times with deionized water, centrifuged and the supernatant was filtered into 100 ml volumetrics and brought up to volume with deionized water. The amount of soluble calcium in the fruit tissue was determined by atomic absorption spectrophotometry as described earlier. The results were expressed as micrograms Ca^{++}/gram fresh weight.

In the acetic acid infiltration study cucumber fruits were submerged in 0, 3, 6, 10, and 15% acetic acid (v/v) solutions. Infiltration procedure was followed as described earlier. Skin (1 mm in thickness) and outermesocarp (2 mm thick flesh next to skin) were dissected and 10 g of each

were blended in 10 ml of distilled water and pH was determined. The infiltrated skin section was extracted and assayed for PME as described earlier.

Effect of blanching with and without $CaCl_2$ on PME activity

To determine the effect of blanching with and without $CaCl_2$, fresh cucumbers (whole) were blanched for 5 min at 75, 87, and 100°C and for 10, 20 and 30 min at 100°C. The effect of $CaCl_2$ on PME activity was determined by blanching at 75 and 100°C for 5 min in solutions containing 0 and 500 mM $CaCl_2$. The whole fruits were blanched in a steam jacketed kettle and constantly agitated during blanching. After heating the cucumbers were cooled by placing them in an ice water bath for 1 min. PME activity of the skin was evaluated under standard assay conditions.

RESULTS

EXPERIMENT I

Localization of PME activity in fresh cucumber fruit (Figure 6) indicates that over 50% of the activity was accounted for in the skin and that 75% occured within the outer 3 mm of the fruit which included the skin and outer-mesocarp. Inner-mesocarp and endocarp account for the remaining 25%. PME activity of a whole cucumber fruit (average sized 3B cucumber equals 50.33 g) was determined by multiplying the average cucumber weight (in grams) by the units of PME activity in micromole/min per gram fresh weight. PME activity in dissected areas was also determined by multiplying the average gram weight of each section by the units of PME activity per gram fresh weight of that particular section. The sum of the activities from the sections (253.4 micromole/min) compares closely to the measured activity of the whole cucumber fruit (224.5 micromole/min). Due to the high concentration of PME activity in the skin area, cucumber skin was used as the crude enzyme source of PME.

The effects of Na^+, K^+ and Ca^{++} concentrations on PME activity are presented in Figures 7, 8, and 9 respectively. Low levels of each of these cations demonstrated stimulatory effects on PME activity. Maximum stimulation of PME occurred at a NaCl concentration of 125 mM, KCl concentration of 200 mM and $CaCl_2$ concentration of 5 mM. These values correspond to 0.73% NaCl, 1.50% KCl, and

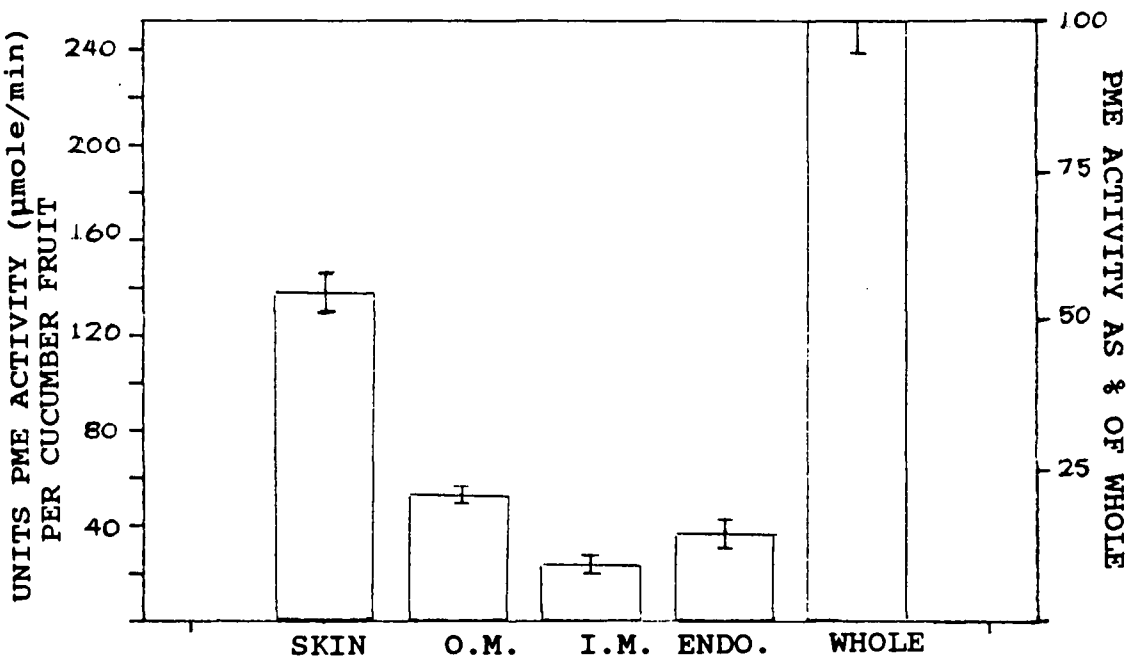

Fig.6 Localization of PME activity in cucumber fruit. Total units of PME activity present in each section of an average sized No. 3B whole cucumber (50.33 g). Each section contributed the following percentage of the whole cucumber weight: skin, 11.34%; outer-mesocarp (O.M.), 27.92%; inner-mesocarp (I.M.), 21.4%; endocarp (ENDO.), 38.68%.

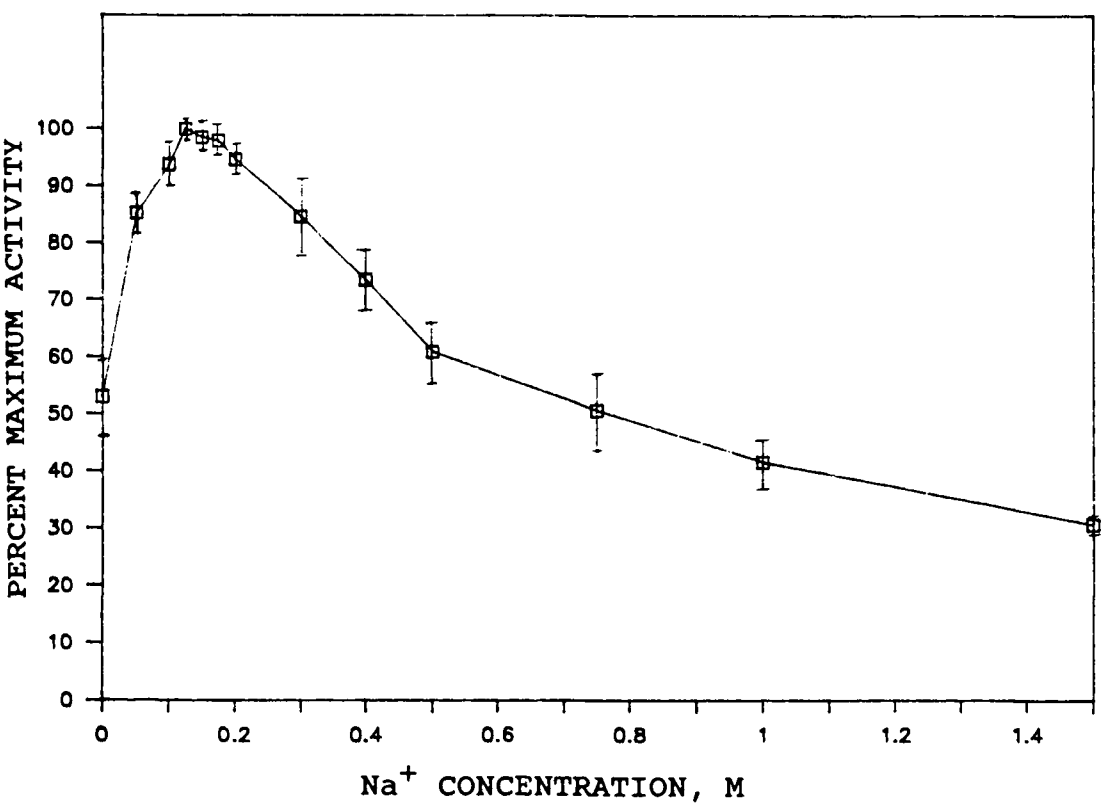

Fig.7 Effect of NaCl concentration on activity of cucumber PME. Reaction mixtures contained 100 µL cucumber PME, 0.5% pectin, and varying amount of NaCl. All reactions were at pH 7.5 and 30°C. 100% maximum activity is equivalent to 10.0 µmole/min/gfw.

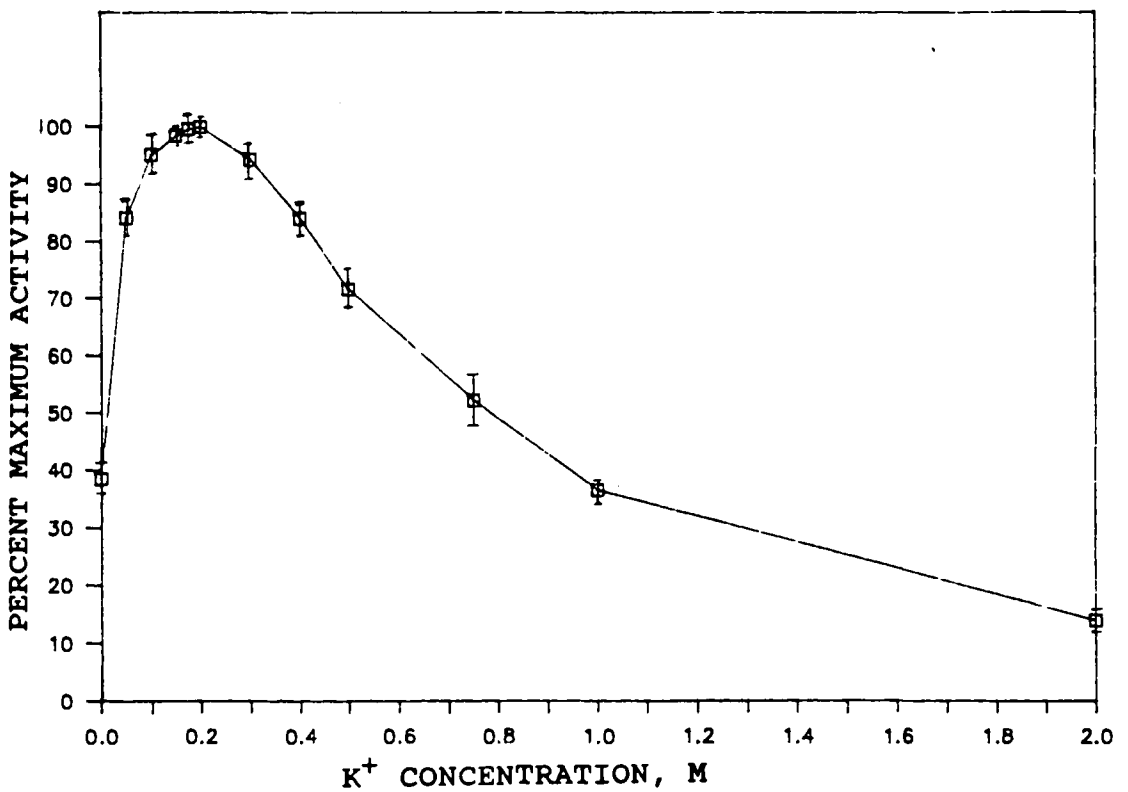

Fig.8 Effect of KCl concentration on activity of cucumber PME. Reaction mixtures contained 100 µL cucumber PME, 0.5% pectin, and varying amount of KCl. All reactions were at pH 7.5 and 30°C. 100% maximum activity is equivalent to 16.7 µmole/min/gfw.

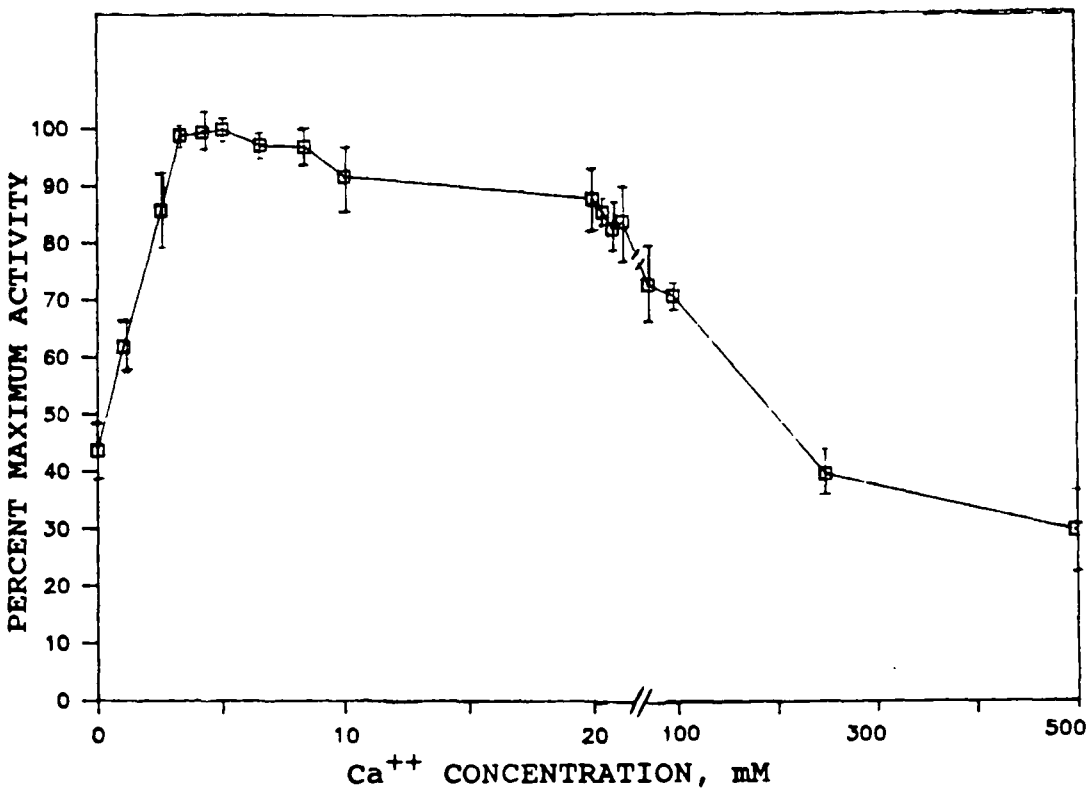

Fig.9 Effect of $CaCl_2$ concentration on activity of cucumber PME. Reaction mixtures contained 100 µL cucumber PME, 0.5% pectin, and varying amount of $CaCl_2$. All reactions were at pH 7.5 and 30°C. 100% maximum activity is equivalent to 10.8 µmole/min/gfw.

0.06% $CaCl_2$. Higher concentrations of each cation were inhibitory.

The optimum activity of PME was observed at pH 8.0 (Figure 10). Deesterification increased rapidly above pH of 5. At pH values above 7.5 beta-elimination or nonenzymatic deesterification of pectin was observed. Deesterification of pectin increased rapidly as pH increased. Figure 10 also shows the difference between total and nonenzymatic deesterification. This plot indicates the deesterification due to PME. At pH 7.5, which was used for standard assay conditions, no measurable beta-elimination occurred. Low and high pH values were inhibitory to PME activity.

The activity of PME increased gradually and reached its maximum at 50°C (Figure 11). Activity droped sharply at 60°C and was completely inactivated at 70°C. The temperature coefficient Q_{10} for PME activity between 10 and 40°C was 1.24.

PME activity was slightly stimulated by increased levels of acetic acid (Figure 12). Optimum activity was observed at 1.5% acetic acid which was 29% greater than PME activity produced at 0% acetic acid. No inhibitory effect on PME was detected by acetic acid levels of 0 to 2.0%.

The results of reversibility of NaCl inhibition of cucumber PME are shown in Table 1. Using 0.15 M as the optimum level of NaCl, 100% maximum activity was defined as the PME activity at this NaCl level. 1.00 M NaCl was

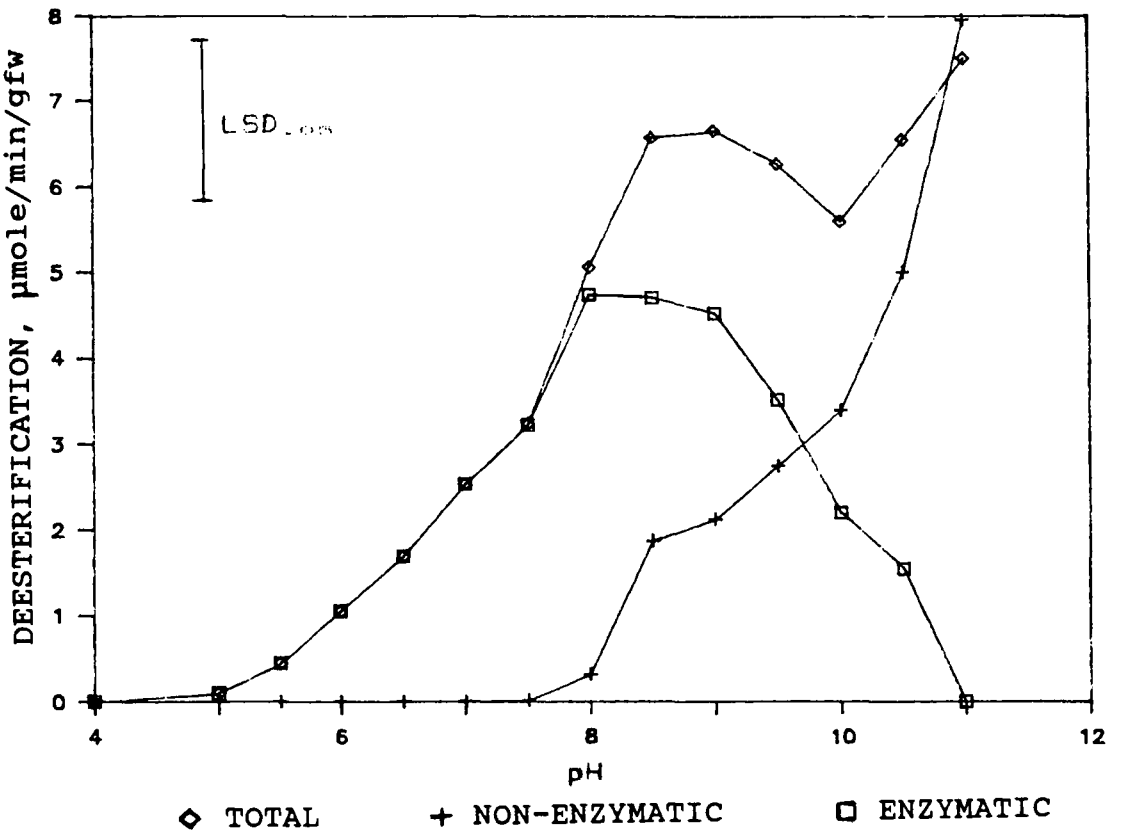

Fig. 10 Effect of pH on activity of cucumber PME. The total reaction mixtures contained 0.5% pectin and 100 µL cucumber PME. Non-enzymatic reaction mixtures contained 100 µL of boiled (5 min. @ 100°C) cucumber PME. The difference between the total and the non-enzymatic reaction mixtures is plotted as the enzymatic deesterification.

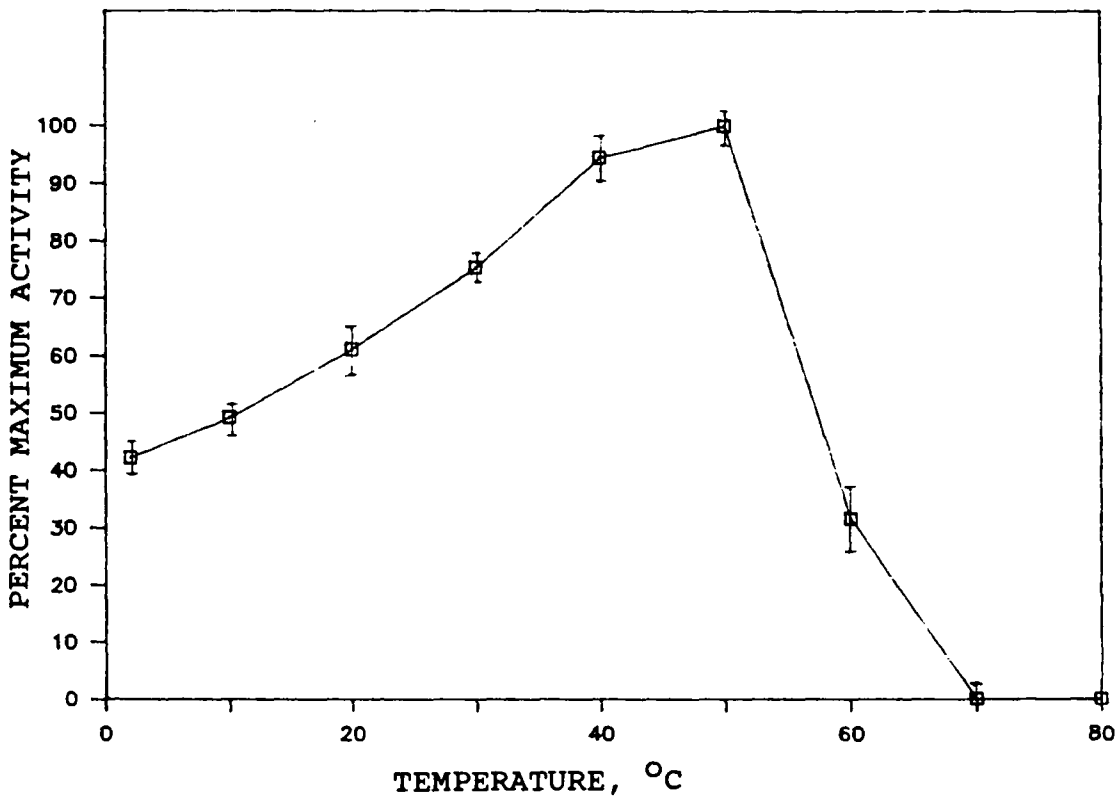

Fig.11 Effect of temperature on activity of cucumber PME. Reaction mixtures contained 100 µL cucumber PME and 0.5% pectin. All reactions were at pH 7.5. 100% maximum activity is equivalent to 5.6 µmole/min/gfw.

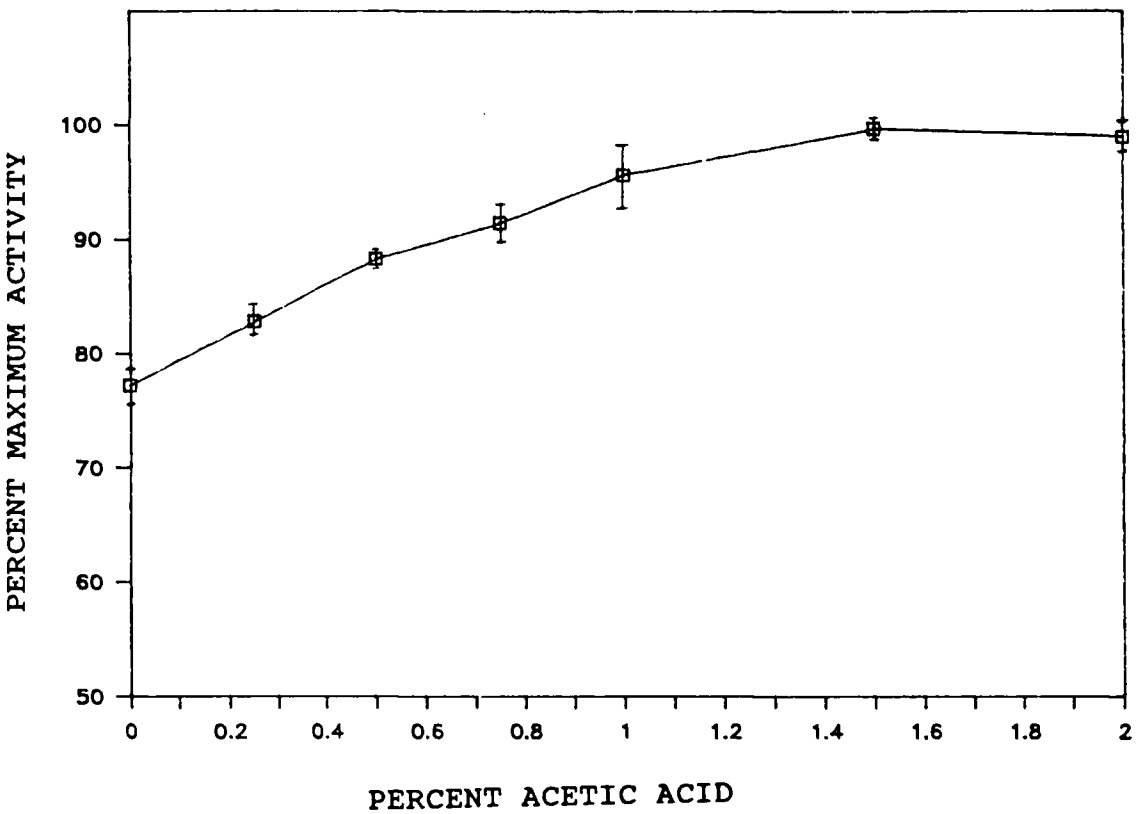

Fig.12 Effect of acetic acid concentration on activity of cucumber PME. Reaction mixtures contained 100 µL cucumber PME, 0.5% pectin, and 363 mM NaCl. All reactions were at pH 7.5 and 30°C. 100% maximum activity is equivalent to 8.3 µmole/min/gfw.

Table 1--Reversibility of NaCl inhibition of cucumber PME[z]

NaCl Concentration (M)		Incubation	%Maximum
Incubation	Assay	Time (hrs)	Activity[y]
-	0.15	0	100.0 a[x]
-	1.00	0	33.6 d
1.00	0.24	24	106.9 a
0.24	0.24	24	71.7 c
-	0.24	0	83.4 b

[z] Cucumber PME was incubated at 30°C at the given NaCl concentrations and incubation times.

[y] 100% maximum activity is equivalent to 4.2 μmole/min/gfw.

[x] Mean separation by $LSD_{.05}$ test; values followed by the same letter are not significantly different ($p \leq 0.05$).

1.00 M NaCl for 24 hours followed by desalting and assaying in 0.24 M NaCl conditions actually increased PME activity slightly indicating that inhibition by NaCl is reversible. Assaying for PME activity in 0.24 M NaCl with or without incubation in 0.24 M NaCl resulted in lower activity as compared to PME incubated for 24 hours in 1.00 M NaCl prior to analysis. PME activity decreased when incubated at the lower NaCl level (0.24 M).

Reversibility of $CaCl_2$ inhibition of cucumber PME is shown in Table 2. Using PME activity at 5 mM $CaCl_2$ as 100% maximum activity, 500 mM $CaCl_2$ reduced activity to 22.3%. PME activity was stable when incubated for 24 hours in both 5 mM and 500 mM $CaCl_2$, suggesting not only that inhibition by $CaCl_2$ is reversible but also that high (500 mM) and low (5 mM) levels of $CaCl_2$ tend to stabilize PME.

EXPERIMENT II

PME activity decreased rapidly during fermentation of cucumbers (Figure 13). Two days after brining, activity was reduced to less than 50% of that present in fresh cucumbers. After 15 days of fermentation, no detectable PME activity remained in cucumbers fermented with or without $CaCl_2$. During the first 6 to 12 hours of fermentation, PME activity in cucumbers fermented without $CaCl_2$ was similar to that found in fresh cucumbers while the presence of $CaCl_2$ in the fermentation brine resulted in rapid reduction in PME activity. After 24 hours there was no difference in PME activity due to the presence of $CaCl_2$.

Table 2--Reversibilty of $CaCl_2$ inhibition of cucumber PME[z]

$CaCl_2$ Concentration (mM) Incubation	Assay	Incubation Time(hrs)	%Maximum Activity[y]
-	5	0	100.0 a[x]
-	500	0	22.3 b
500	5	24	99.7 a
5	5	24	100.2 a

[z] Cucumber PME was incubated at 30°C at the given $CaCl_2$ concentrations and incubation times.

[y] 100% maximum activity is equivalent to 4.6 µmole/min/gfw.

[x] Mean separation by $LSD_{.05}$ test; values followed by the same letter are not significantly different ($p \leq 0.05$).

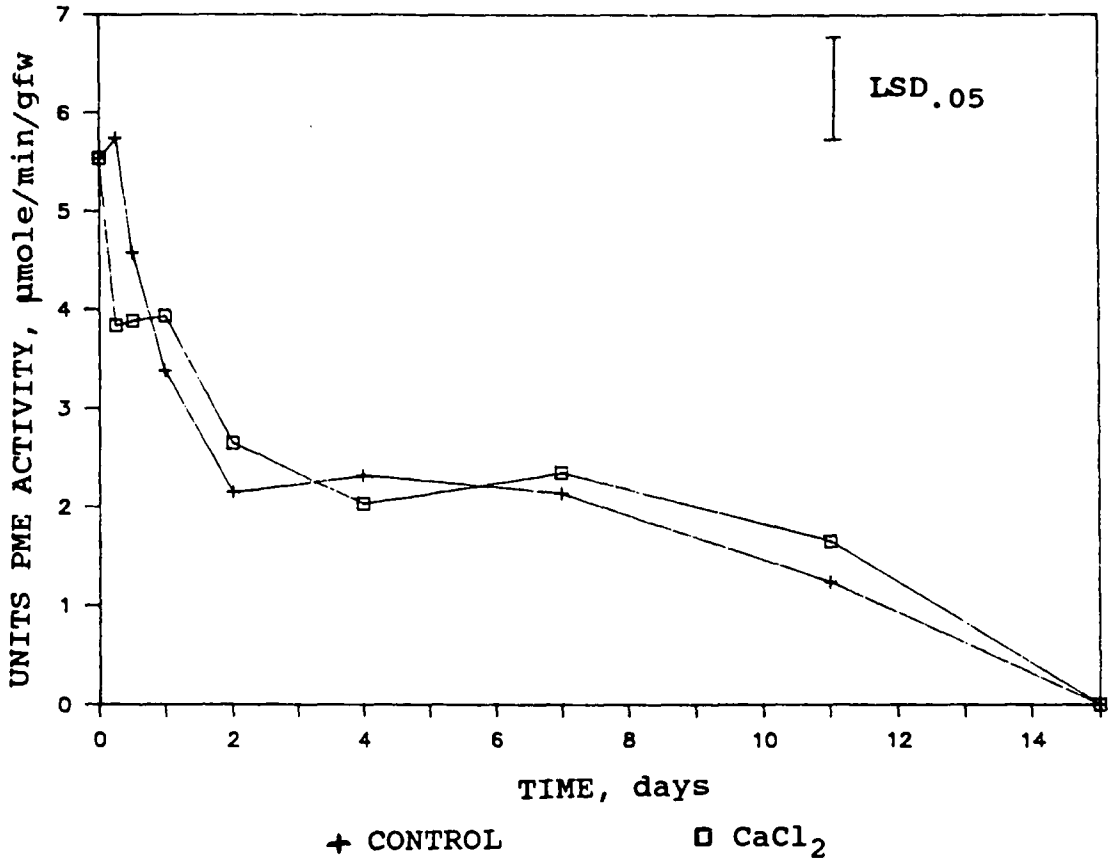

Fig.13 Effect of fermentation with 0 (control) and 100 mM $CaCl_2$ (equilibrated to 40 mM) on activity of cucumber PME. Brine solution contained 4.4% NaCl and 0.1% K-sorbate

The effect of $CaCl_2$ infiltration on PME activity and Ca^{++} content of cucumber tissues is shown in Table 3. While infiltration with $CaCl_2$ resulted in large increases in soluble Ca^{++} content of cucumber tissues (2-7 fold), only slight changes were caused in the distribution and activity of PME in the tissues. A significant reduction in PME activity occurred in the skin tissues accompanied by nominal increases in the other areas of the cucumber. These changes may have resulted from redistribution of PME caused by $CaCl_2$ infiltration, or possibly from slight activation by Ca^{++} in the inner tissues.

Infiltration with increasing concentrations of acetic acid (3-15%) proved most effective in reducing cucumber tissue pH and in inhibition of PME activity (Table 4). With the reduction of skin tissue pH from 6.56 in fresh tissue to 4.10 in skin tissue infiltrated with 15% acetic acid, PME activity in the skin was completely inhibited.

Increasing temperatures (from 75°C- 100°C) during 5 min blanch treatments resulted in greater inactivation of cucumber PME (Table 5). Blanching cucumbers for 5 min at 75°C in the presence of 500 mM $CaCl_2$ caused 20% greater inactivation of PME as compared to blanching without $CaCl_2$. $CaCl_2$ had no effect on PME inactivation in blanch treatments of 100°C. A maximum of 85% inactivation of PME activity was attained even at 100°C for 30 min. This residual PME activity may be due to a thermo-stable form of cucumber PME.

Table 3--Effect of $CaCl_2$ infiltration on Ca^{++} content and PME activity of cucumber tissues.[z]

	Infiltration treatment			
	Control(H_2O)	$CaCl_2$	Control(H_2O)	$CaCl_2$
Cucumber tissue[y]	Soluble Ca^{++} content (µg/gfw)		PME activity[x] (µmole/min/gfw)	
Skin	43	315	4.65a	3.86b
Outer-mesocarp	55	110	0.67ef	0.90dc
Inner-mesocarp	33	128	0.67ef	0.71ef
Endocarp	15	83	0.49f	0.59ef
Whole	33	88	1.30c	1.14cd

[z] Infiltrated treatments consisted of fresh cucumber tissues vacuum infiltrated for 5 min at 150 mm Hg with or without (control) 500 mM $CaCl_2$.

[y] Cucumber tissues were disected to skin (outer 1mm); outer-mesocarp (2 mm next to skin); inner-mesocarp (the space between outer-mesocarp and endocarp); endocarp (locular tissues including the seeds and placenta); and whole fruit without end pieces.

[x] Mean separation by $LSD_{.05}$ test; values between columns and rows are not significantly different if followed by the same letter.

Table 4--Effect of acetic acid infiltration on pH and PME activity of cucumber tissues.[z]

Infiltration Treatment (% acetic acid)	pH		PME activity(skin) (μmole/min/gfw)
	skin	outer-mesocarp	
0	6.56	6.39	1.93
3	6.50	6.31	1.50
6	5.95	6.25	1.03
10	5.35	6.10	0.10
15	4.10	5.50	0.00

[z] Infiltrated treatments consisted of fresh cucumber tissues vacuum infiltrated for 5 min at 150 mm Hg in the designated acetic acid concentrations. Cucumber tissues were disected to skin (outer 1 mm) and outer-mesocarp (2 mm flesh next to skin).

Table 5--Effect of blanch time, temperature, and $CaCl_2$ levels on PME activity of cucumber skin tissue.[z]

Blanch Treatments		PME Activity (% inactivation)[y]	
Time (min)	Temperature (°C)	0 mM $CaCl_2$	500 mM $CaCl_2$
Non-treated (fresh)		0	0
5	75	63	76
5	87	81	-
5	100	83	83
10	100	84	-
20	100	85	-
30	100	84	-

[z] Cucumber skin is defined as the outer 1 mm tissue.

[y] 0% inactivation of PME activity is equivalent to 4.6 µmole/min/gfw.

DISCUSSION

PME is not evenly distributed throughout cucumber fruit. It is primarily concentrated in the skin and outer-mesocarp (outer 3 mm of the fruit). Therefore, partial rather than complete penetration of treatments could be used to control 75% of the PME activity in cucumber fruit.

Cation concentrations for optimum PME activity found in this study (125 mM NaCl, 200 mM KCl, 5 mM $CaCl_2$) are in general agreement with earlier findings (Bell et al., 1951; Lee and MacMillian, 1968; Mayorga and Rolz, 1971; McFeeters et al., 1985; Wills and Rigney, 1979). The studies indicate that PME has definite salt requirements for full activity, and that high salt levels inhibit this enzyme.

The optimum activity of cucumber PME observed at pH 8.0 corresponds with pH optima reported in previous studies on cucumber and other fruits (Bell et al., 1951; Guyer et al., 1956; Kertesz, 1938; Lee et al., 1979; Pressey and Avants, 1972; Van Buren et al., 1962).

The PME activity of cucumber fruit is 25% higher at 50°C than at 30°C. Pozsar-Hajnal and Polacsek-Racz (1975) observed 30% increase from 30 to 50°C in tomato PME activity. The temperature coefficient Q_{10} for PME activity in cucumber fruit determined in this study (1.24) is close to that reported by Bell et al. (1951) for 20 to 60°C (1.33). Q_{10} for PME activity in papaya fruit (1.07) is less than that observed with cucumber (Lourenco and Catutani, 1984), and Q_{10} for PME activity in tomato (1.47) is

slightly higher than that in cucumber (Hills and Mottern, 1947).

Blanching for up to 30 min at 100°C was effective in inactivating only 85% of the PME located in the skin tissue. These results differ from those of McFeeters et al. (1985) who reported complete inactivation at 81°C after blanching for only 3 min. However, these authors also noted a gradual 15-20% reactivation of PME duing 1 month brine storage of blanched cucumbers. McFeeters et al. (1985) suggested that multiple forms of PME may exist, some with thermal stability characteristics. The results of this study support the possible existence of a thermal stable PME in cucumbers. Multiple forms of PME have been isolated from tomatoes, bananas, carrots and oranges, some exhibiting thermal stability (Brady, 1976; Delincee, 1976; Markovic, 1978).

Acetic acid concentrations of 0 to 2.0% were slightly stimulatory in regard to activity of cucumber PME. Addition of a relatively high level of NaCl (363 mM) was necessary to maintain controlled conditions (pH and constant ionic strength) while varying the level of acetic acid. This level of NaCl reduces PME activity to about 80% of maximum and, as a result, some stimulatory and/or inhibitory effects of acetic acid may have been obscured. Since high levels of NaCl are inhibitory to PME, 2% acetic acid was the maximum level that could be studied in vitro.

The most effective method for reducing and inhibiting cucumber PME (in vivo) was infiltration with 15% acetic acid. This treatment resulted in sufficient pH reduction in the skin tissue (to pH 4.10) to completely inhibit PME. This treatment was especially effective for two reasons: (1) pH reduction to 4.10 is outside the functional pH range of PME, thus preventing PME from exhibiting any activity; and (2) treatment of skin tissue with this high concentration of acetic acid irreversibly inhibited cucumber PME in the skin tissue. This second factor is supported by the fact that the acetic acid infiltrated tissue was subsequantly extracted and assayed under optimum pH and ionic strength conditions and no activity was detected.

High levels of NaCl (1 M), though reversibly inhibitory, serve to stabilize the activity of PME, indicating that high NaCl treatments would not be useful as a control method for this enzyme. It was of particular interest to note that PME activity was unstable when incubated at lower NaCl levels (0.24 M) and that activity was stabilized by 5 mM $CaCl_2$ in the absence of NaCl. This stabilizing effect (and reversible inhibition) by $CaCl_2$ suggest that this cation would not be effective as a sole treatment to control PME. However, using inhibitory levels of $CaCl_2$ in combination with another inactivating treatment such as acetic acid infiltration could be beneficial

especially considering the tissue firming effect of Ca^{++} (Bateman, 1964; Grant et al., 1973).

The presence of $CaCl_2$ during brining and fermentation of cucumbers caused a more rapid reduction of PME activity during the first 6 to 12 hours after brining than did brining without $CaCl_2$. The first two days of brining are the most dramatic in terms of rapid pectin demethylation and changes in tissue firmness (Hudson and Buescher, 1985; 1986; McFeeters et al., 1985; Tang and McFeeters, 1983).

Hudson and Buescher (1985) have indicated that addition of $CaCl_2$ to cucumbers at the time of brining was necessary to maintain maximum firmness and higher levels of methylated pectins. The timing of $CaCl_2$ addition was a critical factor since delayed addition of $CaCl_2$ was much less effective in retention of tissue firmness and highly methylated pectins. In a subsequent study, Hudson and Buescher (1986) demonstrated that pectin methylation of cucumber tissue was directly related to tissue firmness. Since demethylation is the result of PME activity, methods of controlling or inhibiting PME may be effective in maintaining firmness in brined and fermented cucumbers. The results of this investigation indicate the most effective method of controlling cucumber PME is pre-brining infiltration of fresh cucumbers with a solution containing 15% acetic acid and 500 mM or more $CaCl_2$.

SUMMARY AND CONCLUSIONS

PME is not evenly distributed throughout cucumber fruit. Over 50% of PME activity was present in the skin and 75% occurred within the outer 3 mm of the fruit. Fermentation study has indicated that the most immediate hours after brining (i.e. 6 to 12 hours) are very crucial. PME activity in cucumbers during the first 6 to 12 hours of fermentation was similar to that found in fresh cucumbers while the presence of $CaCl_2$ in the fermentation brine resulted in rapid reduction in PME activity. Any beneficial treatment should be able to contribute its effect during these important hours. Pre-brining treatment using high concentrations of acetic acid in conjunction with high levels of $CaCl_2$ could be the most effective treatment in controlling PME activity.

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