Original article

Evaluation of enzymatic and non-enzymatic softening in low salt cucumber fermentations

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Summary Retention of a firm, crisp fruit texture is a major consideration for pickled vegetables including pickles made from fermented cucumbers. It is known that cucumbers soften rapidly when fermented at low salt concentrations (< 0.5 M) without added calcium. This study has shown that there is non-enzymatic softening in low salt fermentations because cucumbers soften even when heated sufficiently to inactivate pectinesterase and several glycosidases that can hydrolyse glycosidic linkages that are present in cell wall polysaccharides. Though pectinesterase activity declines and these glycosidases lose activity within the first week of fermentation there is generally greater loss of cucumber tissue firmness when enzymes are not inactivated by heat. While heating cucumbers prior to fermentation reduces softening during subsequent storage, a heat treatment after 2 weeks of fermentation does not reduce softening. This result suggested that the enzymatic reactions responsible for softening occur early in the fermentation process even though the softening does not become evident until later in the storage period. Despite the evidence of an enzymatic component of tissue softening in low salt cucumbers, softening could not be associated with specific enzymes.

Keywords Brining, blanching, *Cucumis sativus*, *p*-nitrophenyl glycosides.

Introduction

Retention of firm, crisp texture during storage and processing is an important quality factor for commercially fermented cucumbers. Approximately 30% of the US pickling cucumber crop is preserved for up to a year by brine fermentation and storage in bulk tanks. Traditionally cucumbers are fermented in 5–8% NaCl. This high salt concentration helps to retain a firm texture of the fruit and also serves as a selection agent for acid tolerant homofermentative lactic acid bacteria, which grow rapidly and convert fermentable sugars to lactic acid and other end products (Fleming *et al*., 1987). After fermentation, salt concentrations may be increased up to 12% or more to reduce the potential for microbial spoilage, and to minimise freezing of the tanks in cold climates in winter. Since finished pickle products contain only 2–4% salt, cucumbers require desalting after fermentation and storage. The brine containing salt and organic matter that is created is a difficult waste disposal issue for the industry. It would be preferable to brine and store cucumbers with sufficiently low NaCl concentrations to reduce or eliminate the necessity for a desalting step. However, it is not possible to assure firmness retention for a year or more when the NaCl concentration is low enough to avoid desalting.

It has been shown that addition of calcium to fermenting cucumbers helps to improve the firmness retention and makes it unnecessary to increase salt levels during post fermentation storage (Fleming *et al*., 1978, 1987; Buescher *et al*., 1979; McFeeters & Fleming, 1991). Fleming *et al*. (1996) demonstrated that pH 3.5 was the optimum pH for assuring microbial stability and texture retention of brine-stock cucumbers at 4% salt. McFeeters *et al*. (1995) determined relationships among different combinations of calcium concentration and pH and the softening rates of fermented cucumber tissue.

The softening that occurs during the fermentation and storage of cucumbers in low salt may be a result of

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enzymatic or non-enzymatic reactions that result in disruption of the cell walls of the fruit. Current evidence indicates non-covalent reactions, such as conformational changes in the cell wall polysaccharides, are most likely to be the cause of non-enzymatic softening (McFeeters & Fleming, 1990; Krall & McFeeters, 1998).

Cucumbers contain enzymes that have been associated with cell wall changes and tissue softening during fruit ripening and in food processing. These include pectinesterase (Bell *et al*., 1951), exo-polygalacturonase (Pressey & Avants, 1975), endo-polygalacturonase (McFeeters *et al*., 1980), cellulase (Miller *et al*., 1989), endo-xylanase (Mujer *et al*., 1991), β-xylosidase (Mujer & Miller, 1991), and an endo-1,4-β-glucanase (Omran *et al*., 1991). Enzymatic tissue softening has been shown to be responsible for economic losses in commercial cucumber fermentations, but this softening was found to be caused by fungal polygalacturonases (Bell *et al*., 1950; Etchells *et al*., 1958) rather than enzymes from the cucumber. Fasina *et al*. (2002) have attempted to improve firmness retention during reduced salt fermentation of cucumbers by using a blanch treatment to inactivate enzymes. However, it has not been shown that cucumber enzymes, which can degrade cell wall polysaccharides, have a significant role in cucumber tissue softening during fermentation and subsequent storage in low salt conditions.

The objective of this research was to assess the relative importance of non-enzymatic and enzymatic activities in causing softening that occurs when cucumbers are fermented and stored with a low salt concentration.

Materials and methods

Fresh pickling cucumbers (size 2A; 25–32 mm diameter) were obtained from a local processor. The fruit were in good condition, not notably desiccated, diseased, or mechanically damaged. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cucumber fermentation

Fermentations with whole cucumbers were done by packing duplicate 46 oz jars (1.36 L) for each sampling time and for each storage treatment with size 2A cucumbers to give a 55:45 pack out ratio (748 g cucumbers: 612 g brine). Whole cucumbers for one treatment with 0.30 M (1.75%) NaCl and no added $CaCl_2$ were blanched at 75 °C for 15 min prior to filling the jars. Brine solutions were prepared that would result in equilibrated concentrations of 1.0 M NaCl, 18 mM $CaCl_2$, 53 mM acetic acid for the control treatment, 0.30 M NaCl, 18 mM $CaCl_2$, 53 mM acetic acid for a reduced salt treatment, and 0.30 M NaCl, 53 mM acetic acid without any added $CaCl_2$, which was expected to soften rapidly. The jars were hermetically sealed by heating caps in boiling water and then closing by hand. A single rubber septum was put in each cap to allow removal of liquid samples during incubation. The day after they were filled and closed, the jars were inoculated with 10^6 CFU mL^{-1} *Lactobacillus plantarum* MOP3:M6. Fermentation and storage of cucumbers was done in a 30 °C incubator. Fermentations were completed in 3 weeks or less based upon a stable pH and lactic acid concentration. After 3 weeks a pair of jars for each sampling time equilibrated with 0.30 M NaCl and no added calcium ion were heated to 75 °C in the centre of the jar and held for 15 min.

To evaluate if slicing cucumbers affected changes in firmness during storage, cucumbers from each fermentation treatment were cut into 6 mm thick slices and repacked into 8 oz jars with the same fermentation brine with a packout ratio of 55% cucumber slices: 45% brine. Texture analysis was done on cucumbers stored both as whole cucumbers and slices.

For experiments in which cucumbers were fermented as slices the cucumbers were first sliced, blanched for 15 min at the target temperature, if required, and then packed into jars and covered with the appropriate cover solution to give a 55:45 cucumber:brine pack out ratio. Duplicate jars were analysed at each sampling time. Inoculation with *Lactobacillus plantarum* culture and storage conditions were the same as described above.

Culture preparation

Lactobacillus plantarum MOP3:M6 was obtained from the culture collection maintained by the Food Fermentation Laboratory, U.S. Department of Agriculture, Agriculture Research Service, Raleigh, North Carolina. This organism lacks the malolactic enzyme that converts malic acid to lactic acid and carbon dioxide. Cultures were maintained at −80 °C in MRS broth (Difco Laboratories, Detroit, MI, USA) containing 16% glycerol. Cultures were streaked onto MRS agar plates and grown for 24 h at 30 °C. Colonies (3–4) of the culture were transferred to 10 mL of MRS broth and grown overnight at 30 °C. Cells were then centrifuged ($5000 \times \boldsymbol{g}$) and washed with 8.5 g L^{-1} NaCl (saline) and re-suspended in 10 mL saline. The culture was plated on MRS medium to determine viable cell numbers. Cells resuspended in saline solution were inoculated into sealed jars to give 10^6 CFU mL^{-1}.

Blanching

Whole cucumbers or cucumber slices were placed in a wire mesh basket and blanched in a steam kettle containing 120–150 L of tap water. Whole cucumbers were blanched at 75 °C for 15 min and cooled in tap water. Slices were blanched at 55, 65, 75, or 85 °C for 6 min depending upon the experiment. After blanching

they were immediately cooled for 6 min in cold tap water. Cucumbers were then drained of excess water and packed. The blanch times were sufficient to raise the internal temperature of the cucumbers or slices to the target temperature (Fasina & Fleming, 2001).

Texture measurement

Firmness measurements were made with a TA.TX2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) using a 3 mm diameter punch. Data were collected and analysed using *Texture Expert* software (Texture Technologies Corp./Stable Micro Systems). The force required to puncture the mesocarp tissue in a cucumber slice was recorded and expressed in Newtons (N). Firmness measurements were done on fifteen cucumber slices from two jars from each treatment and at each sampling time (Thompson *et al*., 1982). Firmness measurements on whole cucumbers were done by taking a single 6 mm thick slice from the centre of each cucumber in a jar.

Extraction of fresh and fermented cucumbers for enzyme activity measurements

All cucumber slices packed in a jar were put in a Waring blender and blended until a homogeneous slurry was obtained. No brine from the jars or additional water was added during blending. Sufficient dry NaCl was added while blending to obtain an equilibrated concentration of 1.0 M NaCl in the slurry. The blended cucumber slurry was filtered through a single layer of Miracloth (Calbiochem, San Diego, CA, USA) and the filtrate centrifuged in a Sorvall (Global Medical Instrumentation, Inc., Ramsey, MN, USA) RC 5B refrigerated centrifuge at 13 000 × ***g*** for 10 min. An aliquot of the supernatant cucumber juice (5–10 mL) was refrigerated and used to assay pectinesterase activity. The remainder of the supernatant juice was placed in 23 mm diameter ×30 cm dialysis bags (~25–30 mL in each bag). The volume of cucumber juice in the bags was first reduced by covering the bags with dry polyvinylpyrolidone (PVP 360). Then PVP was washed off the bags using distilled water and the concentrated juice was dialysed in 0.2 M, pH 5.5 sodium acetate buffer. For 20 mL of fourfold concentrated cucumber juice 2 L of 0.2 M, pH 5.5 sodium acetate buffer was used. The dialysis buffer was changed three times for each dialysis. After dialysis the juice was centrifuged to remove any precipitate.

Enzyme assays

Glycosidase activities were measured by release of *p*-nitrophenol from the appropriate *p*-nitrophenyl glycoside. All commercially available *p*-nitrophenyl glycosidases of sugars present in plant cell walls were tested to determine if they were enzymatically hydrolysed by a dialysed, pH 5.5, 1.0 M NaCl extract from fresh cucumbers. Reaction mixtures (total volume 1 mL) contained dialysed cucumber juice appropriately diluted with 0.2 M, pH 5.5 sodium acetate buffer. Reaction mixtures were equilibrated at 30 °C in a water bath prior to addition of substrate. Reactions were started by the addition of the stock solution of each *p*-nitrophenyl glycoside to the reaction mixture such that the final concentration was 3.0 mM. Samples (200 μL) were taken from a reaction mixture 1, 10, 20 and 30 min after substrate addition. Samples were transferred to a well in a Costar flat bottom, white 96 well plate (COS96fw) (Corning Life Sciences, Lowell, MA, USA) that contained 4 μL of 3 N NaOH to stop the enzymatic reaction and raise the pH to produce a yellow colour due to ionisation of *p*-nitrophenol released from the *p*-nitrophenyl glycoside substrate. The absorbance was measured at 399 nm with a Tecan Saphire plate reader (Tecan US, Research Triangle Park, NC, USA). For each substrate control reactions were done with boiled cucumber extract in 0.2 M, pH 5.5 sodium acetate buffer. Reaction rates were calculated as nanomoles of *p*-nitrophenyl released per minute per gram fresh weight of cucumber based upon an absorbance standard curve for *p*-nitrophenol under the conditions of the assay. For each extract duplicate reactions were done with each substrate.

Extracts from fermenting cucumbers were prepared and assayed from duplicate jars at each sampling time using the same procedure as described for fresh cucumber extracts. Adjustments were made in dilution of the enzyme extracts and in sampling times to take into account the loss of activity that occurred as a result of fermenting or blanching the cucumbers.

Pectinesterase activity was measured based upon the amount of NaOH required to maintain the pH of a pectin solution at pH 7.0 as pectinesterase hydrolysed methoxyl groups from the pectin and released free carboxyl groups from galacturonic acid residues. Rapid set citrus pectin was dissolved in 0.15 M NaCl to give a 0.5% solution. Twenty millilitre of the pectin substrate was put in a 50 mL beaker with a magnetic stirring bar. Two millilitre of the cucumber juice prepared for the pectinesterase assay was added to the beaker. The pH was initially adjusted to 7.0 with 0.1 M NaOH. The pH was then maintained at 7.0 by addition of 0.05 N NaOH as the release of free carboxyl groups by pectinesterase proceeded. Measurement of pH was done with a Fischer Accumet pH meter using a Corning semi-micro combination electrode. Control reactions were done with boiled cucumber extract and substrate in 0.2 M, pH 5.5 sodium acetate buffer with no cucumber extract added. Reactions were done in duplicate.

Polygalacturonase was assayed by the release of reducing groups at 30 °C as a result of hydrolysis of sodium polypectate measured by the procedure of Nelson (1944). Galacturonic acid was used as the standard. A mixture of 3 mL of 0.4 M, pH 5.5 Na acetate buffer and 3 mL of 0.333% Na polypectate was used as the substrate. Dialysed cucumber (4.0 mL) juice prepared for the polygalacturonase assay was added to 6 mL substrate and incubated in a 30 °C water bath. Samples (1.0 mL) were taken at five time intervals (1 min, 2 h 4 h 6 h and 10 h and mixed with 1.0 mL of copper working reagent to stop the reaction (Nelson, 1944). The closed sample tubes were then boiled for 20 min immediately followed by cooling under tap water for 10 min. One millilitre of colour reagent was added, then the samples were centrifuged for 5 min at $12\,000 \times \boldsymbol{g}$ to remove precipitated substrate. Absorbance measurements were made at 600 nm using a Varian Cary Model 300 spectrophotometer (Varian, Inc., Palo Alto, CA, USA). Boiled cucumber extract and substrate in 0.2 M, pH 5.5 sodium acetate buffer with no cucumber extract added were used as controls. Duplicate reactions were run. Reaction rates were calculated as nmoles of galacturonic acid reducing groups released per minute per gram fresh weight of cucumber.

Effect of brine components on glycosidase activities

Fresh cucumbers were extracted with 1.0 M NaCl and prepared for glycosidase activity assays as described above except the cucumber juice was not concentrated with PVP.

Three of the most active glycosidases found in fresh cucumbers, α-D-galactosidase, β-D-galactosidase and β-D-glucosidase, were used to evaluate the effect of NaCl and $CaCl_2$ concentration on inhibiting enzymatic activity. The procedure as described above for glycosidase assays was followed except that combinations of NaCl and $CaCl_2$ were added to 0.2 M, pH 5.5 sodium acetate buffer. Five concentrations of NaCl (0, 0.17, 0.30, 0.60 and 1.0 M) were added to 18 mM calcium chloride. Five concentrations of $CaCl_2$ (0, 18, 36, 100 and 330 mM) were added to both 0.30 M NaCl and 1.0 NaCl. Duplicate reactions were done with each $CaCl_2$, NaCl mixture. These mixtures were prepared by mixing solutions of 0.2 M, pH 5.5 sodium acetate buffer, 0.2 M, pH 5.5 sodium acetate buffer with 500 mM $CaCl_2$, and 0.2 M, pH 5.5 sodium acetate buffer with 1.7 M NaCl in the proper proportions.

Statistical analysis

The ANOVA and GLM procedures of SAS® (Statistical Analysis Systems, Cary, NC, USA) were used for all statistical computations and inferences.

Results and discussion

Effects of low salt and heat treatments on cucumber firmness

Fleming *et al.* (1987) showed that fermentation and storage of cucumbers in 1.0 M NaCl, 18 mM calcium and 53 mM acetic acid would result in fermented cucumbers with commercially acceptable firmness after 1 year or more of storage. These conditions were adopted as the control treatment to evaluate the effect of low salt fermentation and storage and heat treatments on changes in cucumber tissue firmness. Cucumbers for experimental treatments were fermented in only 0.30 M NaCl without added calcium to assure that softening would occur relatively rapidly.

Figure 1 shows changes in the firmness of cucumber slices stored at 30 °C after whole cucumbers were fermented and then cut into slices 4 weeks after the cucumbers were initially brined. As expected, slices stored in 1.0 M NaCl, 18 mM calcium showed only a small loss in firmness during a 9-week storage period. Cucumber slices fermented and stored in 0.3 M NaCl without added calcium showed a large loss of firmness relative to the control. Cucumbers blanched at 75 °C to inactivate enzymes prior to fermentation softened during fermentation and storage compared to the control, but the softening was substantially reduced compared to cucumbers that received no heat treatment. Heating fermented cucumber slices to 75 °C did not reduce softening compared to cucumbers that received no heat treatment. Whole cucumbers stored after the period of

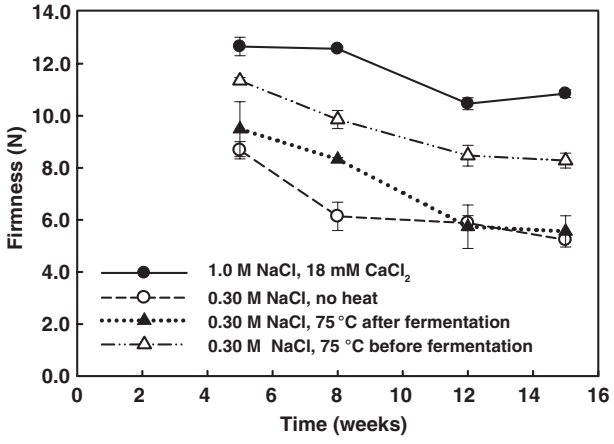

Figure 1 Firmness changes in the mesocarp tissue of cucumber slices after size 2A fruit were fermented with *Lactobacillus plantarum*. Control cucumbers contained 1.0 M NaCl, 18 mM $CaCl_2$ (●). Other treatments contained 0.30 M NaCl without added $CaCl_2$. Cucumbers in treatments with 0.30 M NaCl were not heated (○), heated to 75 °C prior to fermentation (Δ), and heated to 75 °C after completion of the fermentation (▲).

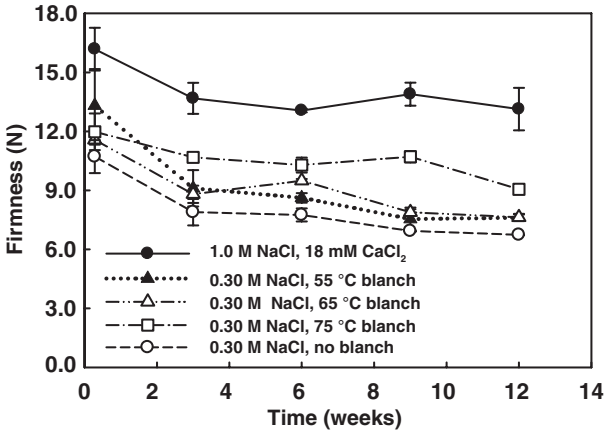

Figure 2 Firmness changes in cucumber mesocarp tissue with different blanch temperatures. Size 2A cucumbers were sliced prior to fermentation and received no blanch treatment or were blanched at 55, 65, or 75 °C and then fermented with *Lactobacillus plantarum* in a brine containing 0.30 M NaCl. In the control fermentation non-blanched cucumber slices were brined in 1.0 M NaCl, 18 mM $CaCl_2$.

active fermentation showed the same pattern of firmness changes as the sliced cucumbers.

Figure 2 shows texture changes in low salt cucumber slices that were blanched at 55, 65, and 75 °C prior to fermentation. There was about a 2 N firmness loss in the control cucumbers during the fermentation period and no further firmness loss during the subsequent 9-week storage period. Slices fermented in 0.30 M NaCl showed an initial firmness loss in the first 2 days after packing cucumbers and a slow reduction in firmness during 12 weeks of fermentation and storage. Firmness retention improved as the temperature of the blanch treatments increased from 55 to 75 °C. The effect of heating cucumbers prior to fermentation on firmness retention indicated that enzymatic activity had a role in softening cucumber tissue in low salt fermentation. However, the fact that cucumber tissue remained much more firm with 1.0 M NaCl and added calcium clearly showed heating was not sufficient to overcome all the softening that resulted from reducing salt and calcium.

Enzymatic activity in fresh cucumbers

The next step was to determine which enzymatic activities that might be involved in cell wall degradation were present in cucumbers. Fresh cucumber extracts were analysed for enzyme activities that might be involved in the degradation of cell wall polysaccharides. Polygalacturonase and pectinesterase measurements were based upon release of reducing groups from polypectate and release of free carboxyl groups from high methoxyl pectin, respectively. Detection of all other enzyme activities was based upon release of *p*-nitrophenol from the *p*-nitrophenyl glycosides. All commercially available *p*-nitrophenyl glycosidases of sugars that occur in cucumber cell walls (McFeeters & Lovdal, 1987) were tested for their ability to be hydrolysed by dialysed extracts from fresh cucumbers even if the glycosidic bond was not one known to occur in plant cell walls.

The cucumber juice extracted with 1.0 M salt did not show hydrolysis of those *p*-nitrophenyl glycosides with glycosidic linkages that are not known to occur in plant cell walls (Table 1, Nos 1–4). Five synthetic substrates with glycosidic linkages known to occur in cell walls were hydrolysed by the cucumber extract (Table 1, Nos 5–9). In addition, *p*-nitrophenyl-α-D-mannoside was hydrolysed (Table 1, No. 10). This linkage is not known to be present in plant cell walls, but has been found in the side chains of glycoproteins (Fry, 1995). Hydrolysis of *p*-nitrophenyl-β-D-mannoside and *p*-nitrophenyl-α-L-

Table 1 Detection of enzymes in cucumbers able to hydrolyse *p*-nitrophenyl glycosides of sugars that occur in plant cell wall polysaccharides

No.	Substrate	Catalog number*	Enzyme detected	Activity detected (+)/(−)
1	*p*-nitrophenyl-α-L-arabinopyranoside	N 3512	β-L-arabinosidase	−
2	*p*-nitrophenyl-α-D-galactopyranoside	N 0877	β-D-fucosidase	−
3	*p*-nitrophenyl-β-D-galactopyranoside	N 1252	β-D-galacturonidase	−
4	*p*-nitrophenyl-β-D-glucopyranoside	N 7006	α-D-glucosidase	−
5	*p*-nitrophenyl-α-D-mannopyranoside	N 2127	α-L-arabinosidase	+
6	*p*-nitrophenyl-β-D-xylopyranoside	N 2132	β-D-xylosidase	+
7	*p*-nitrophenyl-α-L-arabinofuranoside	N 3641	β-D-glucosidase	+
8	*p*-nitrophenyl-β-L-arabinopyranoside	N 0520	α-D-galactosidase	+
9	4-nitrophenyl-β-D-fucopyranoside	N 3378	β-D-galactosidase	+
10	*p*-nitrophenyl-β-D-galacturonide	N 8755	α-D-mannosidase	+
11	*p*-nitrophenyl-α-D-glucopyranoside	N 1377	β-D-mannosidase	−
12	*p*-nitrophenyl-β-D-mannopyranoside	N 1268	α-L-rhamnosidase	−
13	*p*-nitrophenyl-α-L-rhamnoside	N 3513	α-D-xylosidase	−
14	*p*-nitrophenyl-α-D-xylopyranoside	N 1895	α-L-arabinofuranosidase	−

*Sigma-Aldrich Laboratories, Milwaukee, WI, USA.

rhamnoside did not occur to a detectable extent even though these glycoside linkages occur in cell walls (Table 1, Nos 11–12). This may be because these bonds only occur in the main chains of polysaccharides and not as end groups or side chains. Glyosidic linkages of α-D-xyloside and α-L-arabinofuranoside occurred within side chains of cell wall polysaccharides, but no hydrolysis of these *p*-nitrophenyl substrates was observed (Table 1, Nos 13–14). The glycosidase activities observed suggested that several enzymes likely to hydrolyse sugar linkages in cell wall polysaccharides were present in these extracts. All of the *p*-nitrophenyl glycosidase activities detected here have previously been found in cucumbers (Mujer & Miller, 1991; Meurer & Gierschner, 1992), but no estimate of the amount of activity present was reported. All *p*-nitrophenyl glycosidases detected in fresh cucumbers have also been found in 'Bartlett' pears (El-Rayah-Ahmed & Labavitch, 1980) and grapes (Takayanagi *et al*., 1997). Thus, these enzyme activities appear to be widely distributed in fruits and to have the ability to hydrolyse sugar residues from cell wall polysaccharides (Fry, 1995), but their role in plant tissue softening is not clear (Tateishi *et al*., 2005).

In addition to glycosidases capable of hydrolysis of *p*-nitrophenyl glycosidases, pectinesterase and polygalacturonase activities were measured. Pectinesterase activity much higher than the activities of any of the glycosidases in terms of the nmoles per mininute of bonds hydrolysed per gram of cucumber tissue was detected (Table 2). However, polygalacturonase activity could not be detected in the cucumbers used in these experiments even though the same assay method was used as in other investigations of cucumber polygalacturonase (McFeeters *et al*., 1980; Saltveit & McFeeters, 1980). This may mean that current pickling cucumber cultivars have reduced levels of polygalacturonase relative to the low levels of activity that were detected in immature fruit in the late 1970s.

Heat inactivation of enzymatic activities in fresh cucumber slices

In the initial experiments, heating cucumber tissue improved firmness retention. Therefore, it was of interest to determine the heat stability of the enzymes detected in fresh cucumbers. Cucumbers were blanched at 65 and 85 °C and the slurries obtained from the blanched cucumber slices were assayed for pectinesterase and those glycosidases that showed activity in fresh cucumber juice. It was observed that when cucumbers are blanched at 85 °C all of the glycosidases and pectinesterase were inactivated. However, activity of some of the enzymes survived after heating slices for 6 min at 65 °C (Table 2). Four of the six sugar glycosidases lost 60–80% of their activity compared to the activity present in fresh cucumbers. All detectable activity of α-D-galactosidase and β-D-glucosidase was lost after a 65 °C blanch treatment. Pectinesterase was the most heat resistant enzyme among those assayed in that it retained 71% its initial activity after a 65 °C blanch. Heat inactivation of pectinesterase has also been reported by McFeeters *et al*. (1985). It was shown that pectinesterase was inactivated at 81 °C, but not when cucumbers are blanched at 66 °C or less. Meurer & Gierschner (1992) also found that pectinesterase was more heat stable that polygalacturonse because when cucumbers were heated at 65 °C, pectinesterase retained 42% of its activity while polygalacturonase retained only 16% of its activity as compared to non-heated slices. However, when cucumbers were heated at 79 °C, both pectinesterase and polygalacturonase were inactivated completely.

Table 2 Enzyme activities in fresh and blanched cucumbers

Enzyme	Fresh cucumber (nmol min^{-1} g^{-1})	65 °C blanch (% activity remaining)
Pectinesterase	2870 (±410)	70.9
α-L-arabinosidase	0.012 (±0.001)	19.4
α-D-galactosidase	0.63 (±0.10)	<1
β-D-galactosidase	0.82 (±0.12)	38.9
β-D- glucosidase	0.078 (±0.099)	0
α-D-mannosidase	0.60 (±0.06)	30.7
β-D-xylosidase	0.029 (±0.010)	32.9

Effect of ionic strength on activity of glycosidases

Calcium ions greatly reduce the rate of non-enzymatic softening of cucumber tissue (McFeeters & Fleming, 1990). This is presumed to be a result of binding to cell wall polysaccharides whether by cross-linking ionised carboxyl groups in pectin (Grant *et al*., 1973) or by some other binding mechanism (McFeeters & Fleming, 1990). Creation of cross-links among cell wall polysaccharide molecules could reduce the ability of enzymes to diffuse through the cell wall matrix and hydrolyse polysaccharides. In addition, high concentration of ions could inhibit enzyme activities directly. Therefore, the effects of different combinations of NaCl and $CaCl_2$ on the activities of three of the enzymes present in cucumbers, α-D-galactosidase, β-D-galactosidase and β-D-glucosidase, were measured at pH 5.5.

The results in Fig. 3a–c show these enzymes were partially inhibited as sodium and calcium concentrations increased. For the 1.0 M salt, 18 mM calcium concentration that effectively prevented cucumber softening during fermentation and storage, the inhibition was 88%, 56% and 49% for α-D-galactosidase, β-D-galactosidase and β-D-glucosidase, respectively. At equal ionic strength α-D-galactosidase and β-D-galactosidase were inhibited to a greater extent by calcium ions

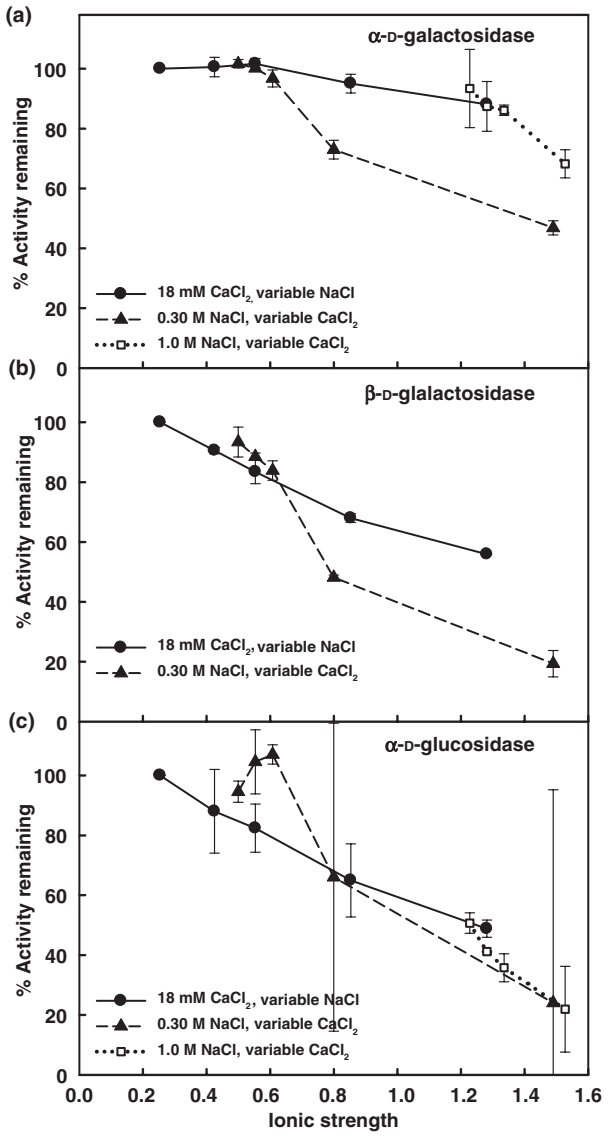

Figure 3 Effect of NaCl and $CaCl_2$ on cucumber α-D-galactosidase (a), β-D-galactosidase (b), and α-D-glucosidase (c) activities at pH 5.5. Activity is expressed as a percentage of the activity in 0.2 M, pH 5.5 sodium acetate buffer.

than sodium ions. However, for β-D-glucosidase the inhibition was dependent primarily on the ionic strength of the solution.

These results show that some inhibition of glycosidases with specificity for glycosidic bonds in cell walls occurs when the ionic strength is high enough to maintain a high degree of tissue firmness. Thus, prevention of softening may in part be the consequence of partial inhibition of glycosidases with high salt brines, ion binding to cell walls that inhibit non-enzymatic softening reactions (McFeeters & Fleming, 1990).

Enzyme and texture changes during cucumber fermentation and storage

Texture and enzyme activity measurements were made during fermentation and storage of cucumber slices. Enzymes were extracted from all cucumber samples with 1.0 M NaCl and then dialysed in 0.2 M acetate buffer, pH 5.5. Therefore, activity differences reflect changes in the amount of active enzyme and not differences caused by variation of the amount of salt during extraction or the amount of NaCl or calcium ions in reaction mixtures.

With all brining treatments, enzyme activities declined rapidly after brining (Figs 4 and 5). Some residual pectinesterase could be detected in cucumbers during fermentation and storage (Fig. 4a). All of the other enzymes assayed had lost all detectable activity after 1 week with the exception of α-D-mannosidase. In the control treatment with 1.0 M NaCl and 18 mM calcium some activity remained after 1 week in one lot of cucumbers (Fig. 4b). This experiment was replicated with a second lot of size 2A cucumbers in which an identical pattern of loss of enzymatic activities was observed with the exception that no α-D-mannosidase activity was observed 1 week after brining the cucumbers (data not shown). To the extent that the six enzymes measured in these experiments are representative of the enzymes in cucumbers that degrade the cell wall polysaccharides, these results suggested that any enzymatic degradation of the cell wall that contributed to firmness loss would occur in the first few days after brining.

Firmness changes during fermentation and storage of the two lots of cucumbers from which enzymes were extracted and activities measured are presented in Fig. 6. For three lots of cucumbers (Figs 1, 2 and 6a), blanching cucumbers before fermentation resulted in some improvement in firmness retention. Since blanching at 75 °C for 85 °C would have inactivated all, or at least most of the enzymes that might degrade the cell wall, the difference in firmness between the higher temperature blanch and non-heated cucumbers must be caused by enzymes in the cucumbers. In the second lot of cucumbers in which enzymatic activities were measured (Fig. 6b) the lack of difference in texture between blanched and non-blanched cucumbers could be a result of a high natural concentration of calcium in the tissue that reduced tissue softening (McFeeters & Fleming, 1989).

The control treatment with 1.0 M NaCl and 18 mM calcium always maintained a substantially firmer texture than any other treatment (Figs 1, 2 and 6a,b). This combination of sodium and calcium chlorides, therefore, must be able to inhibit both enzymatic and non-enzymatic softening. Calcium has been shown to inhibit non-enzymatic softening based upon its ability to lower

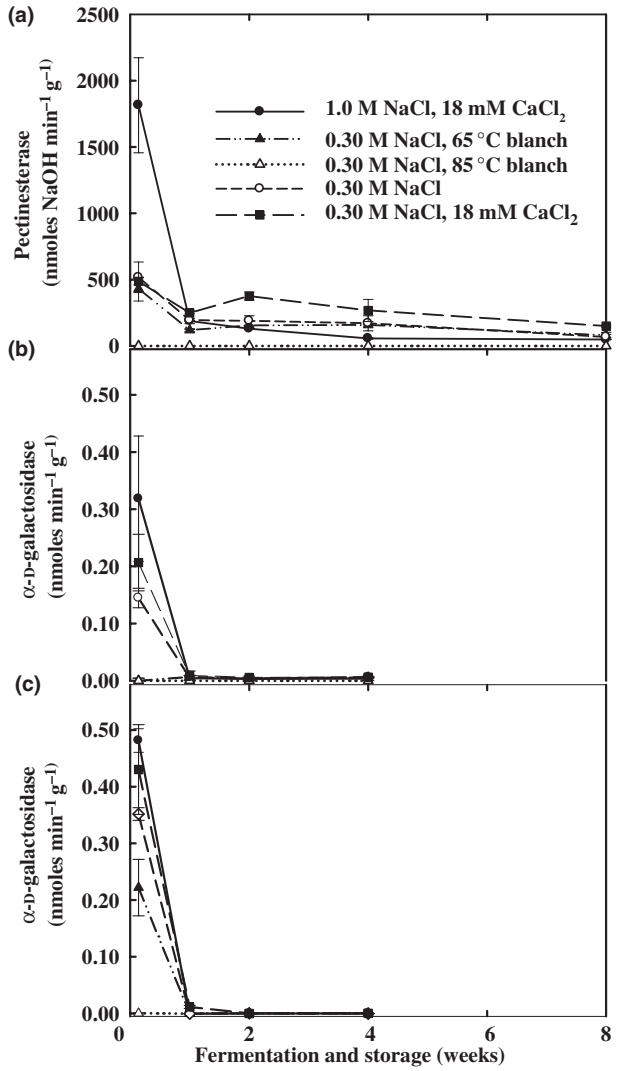

Figure 4 Activities of pectinesterase (a), α-D-galactosidase (b), and β-D-galactosidase (c) in non-heated and blanched cucumber slices during fermentation with *Lactobacillus plantarum* in brine solutions equilibrated with 1.0 M NaCl per 18 mM calcium chloride, 0.30 M NaCl per 18 mM calcium chloride, and 0.30 M NaCl.

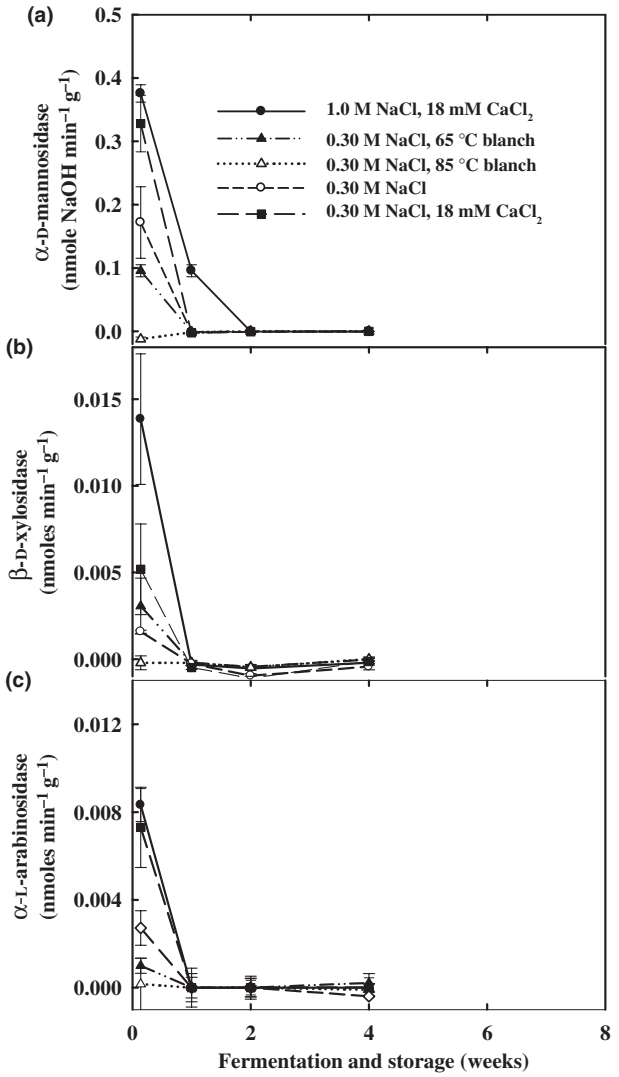

Figure 5 Activities of α-D-mannosidase (a), β-D-xylosidase (b), and α-L-arabinosidase (c) in non-heated and blanched cucumber slices during fermentation with *Lactobacillus plantarum* in brine solutions equilibrated with 1.0 M NaCl per 18 mM calcium chloride, 0.30 M NaCl per 18 mM calcium chloride, and 0.30 M NaCl.

the entropy of activation sufficiently that it raises the free energy of activation of the reaction (McFeeters & Fleming, 1990). However, salt clearly must contribute to the prevention of non-enzymatic softening, since the only difference between the 0.30 M NaCl, 18 mM $CaCl_2$ and the control fermentations was 0.70 M NaCl (Fig. 6a,b).

These results show some benefit for blanching cucumber tissue to reduce texture loss, but the improvement is not sufficient to maintain adequate firmness with low salt and calcium concentrations. The ability of high concentrations of calcium to inhibit enzymatic activity equal to or better than NaCl at the same ionic strength (Fig. 3) as well as the ability of low calcium concentrations to reduce softening, raises the possibility that brining cucumbers at higher concentrations of $CaCl_2$ than the 18 mM used here might provide a means to maintain cucumber firmness while substantially reducing waste generation. Even if the concentration of calcium required were so high that some removal of calcium from the fermented cucumbers would be necessary, disposal of brines containing calcium chloride would not harm water and soils as disposal of NaCl does. In areas where soils are deficient in calcium, such

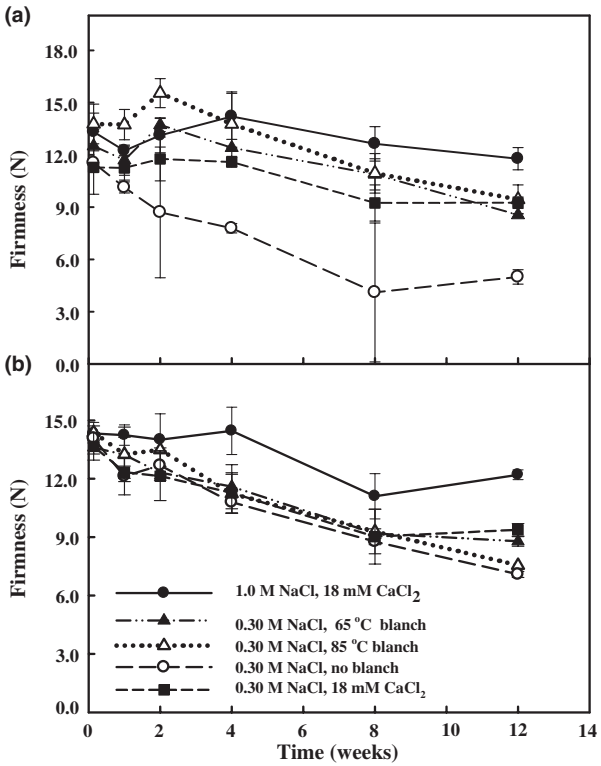

Figure 6 Firmness changes during fermentation and storage of size 2A cucumbers from which enzymes capable of hydrolysing methyl ester groups from pectin or *p*-nitrophenyl glycosides of sugars present in plant cell walls were extracted (Figs 4 and 5). Control cucumbers contained 1.0 M NaCl, 18 mM $CaCl_2$ (●). Other treatments contained 0.30 M NaCl with or without added $CaCl_2$ as indicated in panel B. Slices stored in 0.30 M NaCl either received no heat treatment or were blanched at 65 °C or 85 °C.

as the costal plain of North Carolina, addition of calcium containing brines to soils may be beneficial.

Conclusions

Fermentation of cucumbers in 1.0 M NaCl plus 18 mM calcium maintained firmness better than any treatment with reduced salt. Heat treatment of cucumbers sufficient to inactivate cell wall degrading enzymes before fermentation helped reduce firmness loss during fermentation and storage. However, heat treatment of cucumbers after fermentation did not prevent firmness loss during storage at 30 °C. This suggested that reactions that resulted in softening of the cucumber tissue during storage had occurred during the fermentation period and that heating after fermentation could not prevent those changes. Glycosidases and pectinesterase rapidly lost activity at the beginning of the fermentation process. However, textural changes related to enzymatic mechanisms may not be fully evident until later in the storage period.

The effect of heating cucumbers prior to fermentation on firmness retention indicated that enzymatic activity had some role in softening cucumber tissue in low salt fermentation. During fermentation of cucumbers that are not blanched, activities of certain glycosidases like α-D-galactosidase, β-D-galactosidase and β-D-glucosidase may be partially inhibited by high ionic strength in the fermentation brine caused by the presence of high NaCl and calcium. In addition to reducing the activity of cucumber enzymes early in the fermentation, cross-linking of cell wall polysaccharides by sodium and calcium ions might also restrict the ability of enzymes to attack cell wall polysaccharides and thereby prevent softening. Since calcium chloride may be more effective in preventing softening at lower concentrations than NaCl, fermentation of cucumbers in calcium chloride without the use of NaCl should be considered.

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