pH Effect on Calcium Inhibition of Softening of Cucumber Mesocarp Tissue

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ABSTRACT

The first-order rate of softening of cucumber tissue containing 1.5M NaCl with and without 20 mM added calcium ion was determined from pH 2.5 to 8.6 at 74°C. Calcium ion had little inhibitory effect on rate of softening when pH was above 5. Below pH 5 the relative effectiveness of calcium in reducing rate of softening increased as pH decreased. This behavior was opposite to that expected if calcium inhibited softening by cross-linking negatively charged pectin carboxyl groups. This was more evidence against that mechanism as an explanation for inhibition of softening by calcium ion in plant tissues. A very large decrease in rate of tissue softening was observed at alkaline pH whether or not calcium was added.

INTRODUCTION

EFFECTS OF pH on loss of firmness of plant tissues have received a limited amount of attention over the years. Available data indicate that softening is rapid in neutral and very acid conditions with minimum softening in the region of pH 4 (Mattson, 1946; Doesburg, 1961). However, combined effects of pH variation and calcium addition have not been investigated. A very large effect of calcium in inhibiting rate of cucumber mesocarp softening at a pH of 3.2-3.5 has been reported (McFeeters and Fleming, 1989; 1990). Multiple lines of evidence based on the ability of various metal ions to affect the softening rate (McFeeters and Fleming, 1989) have led to the proposal that calcium does not inhibit softening by pectin cross-linking according to the "egg box" model of Grant et al. (1973). The primary idea of the model was that negatively charged polypectate molecules form a gel in the presence of divalent, positively charged calcium ions because the calcium ions fit into ionic sites and cross-link pectate molecules to give a three-dimensional gel structure. The pK of pectin carboxyl groups is about 3.6 (Cesaro et al., 1982). The requirement of ionized carboxyl groups for calcium binding means that maximum softening inhibition by calcium should occur near pH 6, where nearly all free carboxyl groups would be negatively charged. As the pH is lowered, calcium should become relatively less inhibitory due to loss of negative charges as carboxyl groups become protonated. The main objective of our study was to determine the effect of calcium on softening rate of cucumber tissue as a function of pH to test this prediction of the egg box model. A second objective was to determine the pH range that would minimize loss of firmness for processing of pickled cucumber products with and without added calcium.

MATERIALS & METHODS

SIZE 3B CUCUMBERS (45-51 mm diameter) were obtained from a local processor. Cucumber mesocarp tissue pieces were prepared by peeling the fruit and cutting them into 7 mm thick slices (McFeeters et al., 1989). About 15 mm of tissue from both stem and blossom ends of the fruit were discarded. Mesocarp pieces were cut from the three carpels of each slice. The seed area was discarded. Mesocarp pieces (400 g/batch) were blanched for 3 min in 13 L boiling distilled water and cooled for 2 min in cold distilled water. Previous work has shown that this blanch treatment inactivated pectinesterase (McFeeters et al., 1985), so that pectin methylation would be about 60% (McFeeters et al., 1989; McFeeters and Fleming, 1989). The blanched pieces (30 ± 0.5 g/jar) were packed into 60 mL (2 oz) jars and covered with a volume of brine equal to the weight of cucumber tissue. The jars were stored at 4°C for at least 24 hr prior to heating to allow equilibration of the brine with the cucumber tissue. Twelve jars of each treatment were placed in a 74°C water bath. After 10 min for temperature equilibration, two jars were removed, cooled rapidly in a circulating 25°C water bath, and the firmness measured. The mean firmness of the tissue in these jars was recorded as the initial firmness. Duplicate jars were sampled at subsequent times and the firmness measured by a punch test on 15 tissue pieces (Thompson et al., 1982). Due to large differences in softening rates as a function of pH and calcium, the time period over which samples were taken varied from 20 min for rapidly softening tissue to 24 hr when softening was very slow.

The brine solutions had to be prepared such that after equilibration of the cucumber tissue with the brine solutions and with the temperature at 74°C, the intended pH was achieved. To maintain the intended pH values, the brine solutions need to be adequately buffered over the pH range of interest. A stock brine solution of 3.0M NaCl, 200 mM Na acetate (pK = 4.7), 40 mM HEPES (pK = 7.5), and 40 mM CHES (pK = 9.3) was prepared. An Orion 901 pH meter with an Orion combination electrode designed to operate at temperatures up to 80°C was standardized with phosphate buffer (pH = 6.986 at 74°C) and potassium hydrogen phthalate (pH = 4.141 at 74°C) buffer (Dawson et al., 1986). Blanched cucumber mesocarp tissue was blended. One part of the blended slurry (30g) was mixed with 30 mL of the stock brine solution and titrated to pH < 2 with 3.017M HCl. A second sample prepared in the same way was titrated with 3.032M NaOH to pH > 9. The temperature was maintained between 73.2°C and 75.0°C during titrations. Based on the titrations, the appropriate amount of HCl or NaOH was added in the stock brine solution. To investigate the effect of added calcium, 40 mM $CaCl_2$ was added to a second solution at each pH. Twelve jars of each treatment were prepared. The intent was to have cucumber mesocarp tissue equilibrated with and without 20 mM added $CaCl_2$ with pH values near 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, and 9.0. The pH value achieved was determined by measuring pH of the brine at 74°C from two jars of each pH treatment after firmness of tissue pieces had been determined. The average pH difference between duplicate jars was 0.011 with a range from 0 to 0.078 over all treatments.

Calcium analysis

Samples were prepared by blending cucumber tissue with an equal volume of 4% trichloroacetic acid to release calcium from the tissue. Calcium was analyzed by the colorimetric procedure for Gindler and King (1972). An automated version of this procedure has been shown to give results equivalent to atomic absorption analysis for peanut samples (Salazar and Young, 1984).

Data analysis

First-order softening rate constants (Huang and Bourne, 1983) were calculated by linear regression analysis using the mean tissue firmness calculated for each sample jar as a function of time.

RESULTS

THE NATURAL CALCIUM concentration in cucumber tissue has been observed to have a large effect on the rate of cuc-

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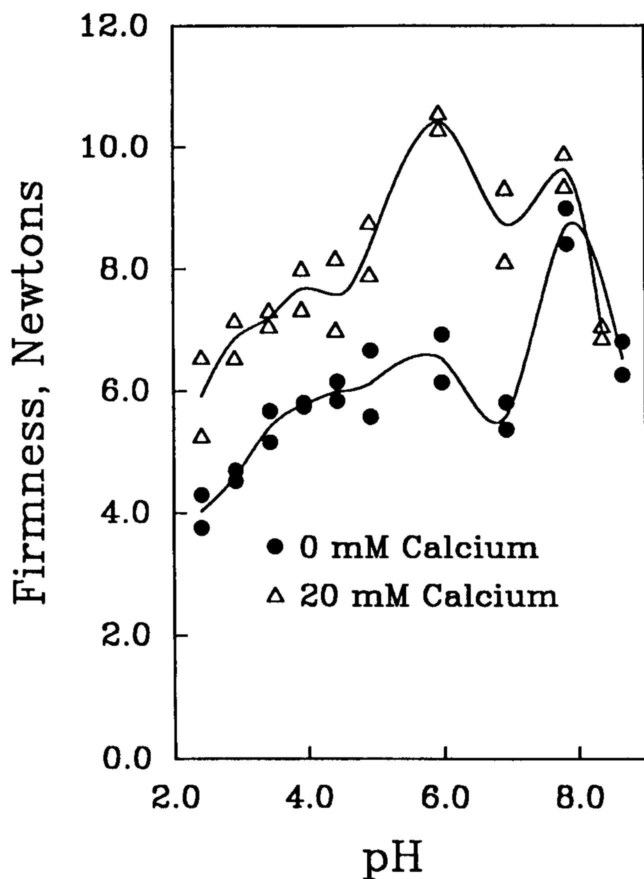

Fig. 1—Firmness of cucumber mesocarp tissue after equilibration of tissue in 0.1M acetic acid, 40 mM HEPES, 40 mM CHES, and 1.5M NaCl with and without 20 mM added $CaCl_2$ at 4°C. Temperature of samples raised to 25°C prior to firmness measurement.

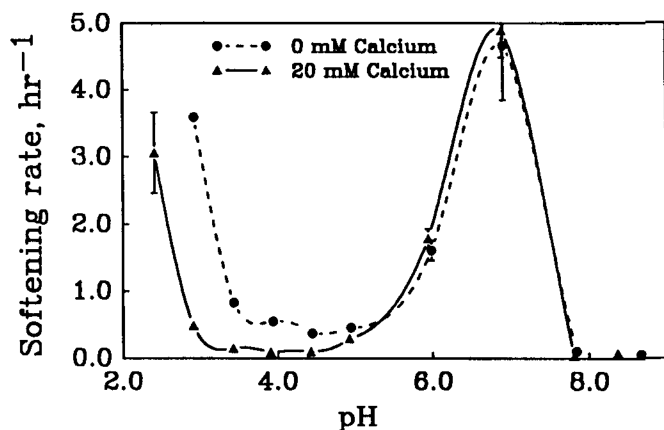

Fig. 2—First-order softening rates of cucumber mesocarp tissue in 0.1M acetic acid, 40 mM HEPES, 40 mM CHES, and 1.5M NaCl with and without 20 mM added $CaCl_2$. Bars indicate standard deviation of softening rate. Where bars not visible, standard deviation of softening rate was within range of circles on graph. Incubation temperature 74°C. Samples cooled to 25°C prior to firmness measurement.

umber tissue softening (McFeeters and Fleming, 1989). The blanched cucumber tissue contained 2.9 mM calcium ion, a relatively low level of natural calcium. Figure 1 shows the firmness of mesocarp tissues upon removal from the 4°C equilibration period. The samples to which 20 mM calcium was added were always firmer than those with no calcium. With exception of the highest pH, the firmness tended to increase as pH increased. These results represent the initial firmness of the tissues prior to incubation at 74°C. At pH 2 without added calcium, the cucumber tissue had softened to such an extent during 24 hr in the refrigerator that it was not possible to measure the rate of softening.

The 74°C temperature was chosen for these experiments for two reasons. First, it was not necessary to use a preservative to prevent microbial growth in the samples due to the high temperature and short incubation times (24 hr or less). Previous work has been done at 44°C using 3 mM sulfite as a microbial inhibitor (McFeeters et al., 1989; McFeeters and Fleming, 1989). However, sulfite is not effective at neutral or alkaline pH. Secondly, 74°C is the recommended internal temperature for pasteurization of fresh-pack cucumber pickles (Monroe et al., 1969).

Softening rates as a function of pH are shown in Fig. 2. If we first consider the curve without added calcium, at neutral pH the softening rate was very rapid. Rate of softening declined dramatically at both alkaline and acid pH. Once the pH was adjusted below 4, softening rates again became very rapid. When 20 mM calcium was present in the tissue, the general pattern of softening rate changes, as a function of pH, was similar to that without calcium. However, calcium had an increasing relative effect in inhibiting rate of softening compared to the tissue without added calcium as the pH was lowered. This relationship is illustrated in Figure 3 which shows the ratio of the first-order softening rates without and with added calcium as a function of pH. A ratio > 1 indicated that addition of 20 mM calcium inhibited rate of softening.

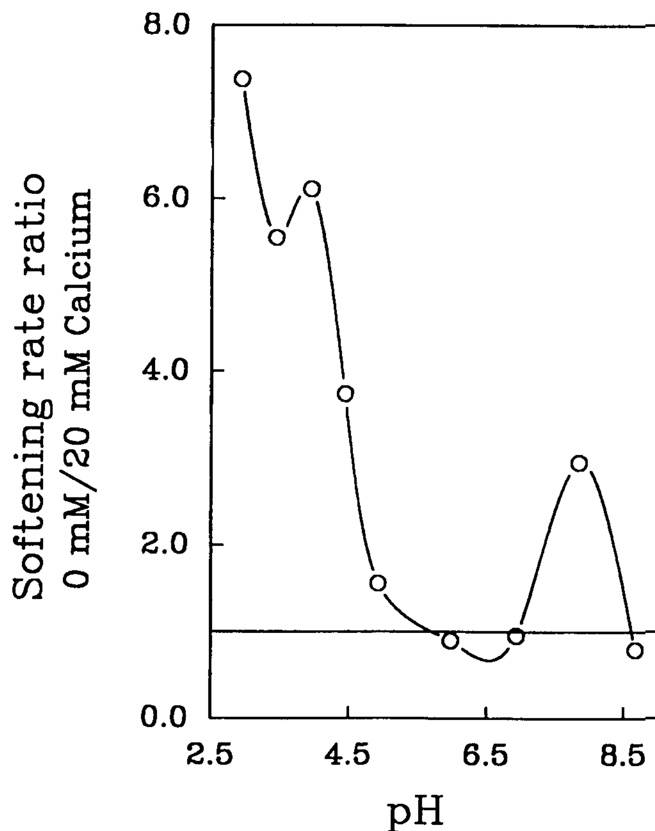

Fig. 3—Ratio of first-order softening rates as function of pH. Softening rates without added $CaCl_2$ were divided by softening rate at same pH with 20 mM added $CaCl_2$.

DISCUSSION

THIS IS THE FIRST REPORT of first-order rates for plant tissue softening as a function of pH. By using a high ionic

strength, the possibility has been eliminated that changes in firmness previously attributed to the effect of pH may, at least in part, have been due to differences in ionic strength of the buffering ions as the pH was changed (Doesburg, 1961). The added NaCl made a contribution to the ionic strength of 1.5. At a given pH, the difference in ionic strength due to addition of 20 mM $CaCl_2$ was 0.06. The ionic strength contribution from buffer ions and unknown ionic species in the cucumber tissue would be identical at a given pH. Over the pH range studied, the difference in ionic strength due to ionization of the buffer species was less than 0.10.

In the absence of added calcium, the pattern of changes at neutral and acidic pH was similar to that reported for turnips, potatoes, beets, apples, and cauliflower tissue pieces in sodium citrate solution using a single point analysis after heating at 100°C for 10 min (Doesburg, 1961). Improvement of firmness retention by lowering the pH of low acid tissues has also been reported for white potato (Hughes et al., 1975), sweet potato (Sistrunk, 1971), and carrot (Heil and McCarthy, 1989).

In the alkaline pH range there was a remarkable decline in rate of softening. The only previous report of the structural result of exposure of plant tissue to alkaline pH was that of Ginzburg (1961). He visually estimated the disintegration of pea root tips exposed to solutions of different pH. He observed a decline in disintegration over the range of pH 7 to 10. Thus, to the extent that comparison can be made given the differences in measurement, results appear similar.

The main objective of our work was to determine the effect of calcium on softening rate of mesocarp tissue as a function of pH to test the egg box model for calcium action. Figures 2 and 3 show that calcium had little effect as an inhibitor of softening rates above pH 5. Some inhibition by calcium was observed at pH 7.8, but the significance of that point is uncertain since no inhibition was observed at pH values immediately above or below 7.8. As indicated by the increasing ratio of softening rate without added calcium compared to the rate with 20 mM calcium as the pH declined below 5 (Fig. 3), the relative effect of calcium increased rapidly. If the egg box model (Grant et al., 1973) was the primary mechanism responsible for calcium inhibition of softening rates, the prediction would be that the relative effect of calcium should increase as the pH increased due to the increased number of ionized carboxyl groups to provide more sites for calcium cross-links. Since the opposite was observed experimentally, this result provided more important evidence against the egg box model of calcium action as a mechanism to inhibit tissue softening. Other arguments against the egg box model have been made previously (McFeeters and Fleming, 1989; 1990). From a practical point of view, our result suggests that the importance of calcium as a means to retain firmness of cucumber tissue will increase as the pH of the product decreases. It also suggests better firmness retention may occur if cucumber tissues are acidified prior to heat-processing. Previous indications that calcium might be more effective in inhibiting firmness loss in plant tissues at a lower pH were reported by Hughes et al. (1975) and Thompson et al. (1979). A greater retention of firmness was found in fermented cucumbers when calcium was added at pH 3.3, as compared to pH 3.8 Thompson et al. (1979). Over the limited pH range of 4.6 to 6.2, Hughes et al. (1975) observed somewhat firmer potato disks at lower pH when calcium was added to cooking water.

Given the rather complex relationship between pH and the rate of cucumber tissue softening, what type of chemical mechanisms might be involved in softening? Traditional ideas have focused upon degradation of polysaccharides by hydrolysis or β-elimination (Doesburg, 1965). The explanation for the observed rate profile would be that at low pH there is degradation by acid hydrolysis. The minimum softening rate near pH 4 occurs because both acid hydrolysis and β-elimination are very slow at that pH. The rate of softening increases as pH 7 is approached due to increasing β-elimination rates. Finally, above pH 7, β-elimination would be greatly slowed due to demethylation of the pectin in the alkaline region (Albersheim et al., 1960). β-Elimination has been shown to occur in solutions of pectin and isolated cell wall suspensions. There are, however, some problems. First, β-elimination has not been demonstrated to occur during heat-softening of intact plant tissues. Most recently, Platt et al. (1988) were unable to find evidence for β-elimination in blanched carrot tissue. Also, differences in ionic strength, buffer composition, and temperature between this experiment and the recent work of Sajjaanantakul et al. (1989) on the β-elimination reaction were great. Still, it appears that softening of cucumber tissue was considerably faster than might be expected if β-elimination were the primary degradative reaction in the pH 4 to 7 range. There is also some question about assignment of acid hydrolysis as the rate-limiting step for softening at low pH. An analysis of the thermodynamics of cucumber tissue softening at pH 3.3 suggests that the rate-limiting step is unlikely to be a hydrolytic reaction at that pH (McFeeters and Fleming, 1990). The enthalpy and entropy of activation in the absence of added calcium are both much larger than would be expected for hydrolysis of glycosidic bonds. They are more like values for protein denaturation or some other reaction involving conformational change. So while the softening pattern can be rationalized on the basis of the known chemistry of pectin, recent data suggests some caution in assignment of these mechanisms to explain plant tissue softening. It would be of interest to determine the thermodynamic parameters in different pH ranges for an indication of where changes in the softening mechanism occur.

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Detoxification using an expeller with and without added ammonium hydroxide

The normal expelling of aflatoxin-containing copra did not reduce aflatoxin level in the copra residue (Table 5). There was 33% reduction in the oil which might have been due to the heat generated during expelling. The temperature of the oil at the surface of the expeller bars reached a maximum 70°C. The lowest limit for temperature-induced breakdown of aflatoxin B_1 was around 60°C (Mann et al., 1967). The thermal detoxification of aflatoxin may have been more pronounced in the oil than the residue because the oil was a better thermal conductor than the solid residue.

Addition of ammonium hydroxide (1% NH_3) before expelling did not reduce the aflatoxin level of the residue; however, a higher reduction was observed in the oil. The aflatoxin breakdown in the oil could have been due to the increased solubility of ammonia combined with the detoxifying effect of heat. Time in the expeller was less than 15 min, thus there was not much time for chemical interaction of ammonia with copra. Grehaigne et al. (1983) on their study of extrusion-cooking of aflatoxin-containing peanut meal suggested that an increase in residence time in the extruder could result in greater reduction of aflatoxin. Moreover, the volatilization of ammonia during expelling further reduced the detoxification. Thiesen (1977) attempted detoxifying groundnut meal with ammonia during pelleting and reported almost all the ammonia was driven off during the operation.

Reversibility test

The possibility of reversion of ammoniated copra aflatoxin converted by-products in copra (aflatoxin content of <20 ppb) to the original aflatoxin molecule after acidification was tested. There was no change in aflatoxin content of copra before and after acidification (pH 2, 37°C, 5 hr). Therefore, acidification simulating upper gastrointestinal tract conditions did not revert the ammoniated products to aflatoxin B_1. Similar studies on the effect of acidification of ammoniated commodities, e.g. cottonseed, peanut meal, corn, using the APAT procedure showed stability of the conversion by-products (McKinney et al. 1973; Prevot and Jemmali, 1977; Schroeder et al., 1985).

However, proteolytic hydrolysis of the detoxified substrate should be carried out to establish whether the aflatoxin-protein interaction could be broken down by proteolytic enzymes and thus reverted to the original aflatoxin molecule. Park et al. (1982) reported a significant amount of aflatoxin B_1 (12%) and aflatoxin-related compounds (27%) were liberated from the detoxified residue (monomethylamine/lime-treated) following enzyme (pronase) digestion.

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