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Effects of NaCl on Fermentative Metabolism of Mature Green Tomatoes cv. Ailsa Craig in Brine

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Summary

The effect of osmotic strength on gene expression and activity of the major enzymes of fermentative metabolism of mature green tomato fruit (*Solanum lycopersicum* cv. Ailsa Craig) has been studied by exposing fruit to brine containing 0 (water), 5 and 10 % NaCl. The fruits were surface sterilized prior to treatment to prevent the growth of microbes naturally present on the skin of the fruit. Changes in fruit expression of fermentation genes and the activity of the respective enzymes as well as physicochemical quality characteristics (soluble solid content, titratable acidity, pH and firmness) were studied in both fruit and brine for 0.5, 1, 1.5, 2, 3, 7 and 14 days. Discrepancies in responses that resulted from the different salt concentrations were obtained at molecular and quality levels. The complex kinetics of solutes between the fruit and the surrounding solution due to osmotic potential has led to different responses of the tissue to fermentation. Tomato fruit showed cracking soon after storage in water; water-stored fruit had higher titratable acidity, lower soluble solid content, and higher induction of anaerobic metabolism as indicated by the expression or the activity of the fermentation enzymes compared to fruit stored in brine with 5 or 10 % NaCl. No cracking was observed in fruit stored in 5 (isotonic) or 10 % NaCl (hypertonic) brine, though in the latter, signs of dehydration were observed. The presence of salt in brine reduced the intensity of fermentative metabolism as indicated by the lower gene expression and enzyme activity. However, fruit stored in brine with 5 % NaCl survived longer than with 0 or 10 % NaCl. The presence of 5 % NaCl in brine caused mild changes of both the fermentative metabolism and the physicochemical characteristics and prevented fruit deterioration during storage.

Key words: alcohol dehydrogenase, pyruvate decarboxylase, lactate dehydrogenase, anoxia, tomato fruit

Introduction

Unripe (green) tomatoes, though not as popular as the ripe (red) ones, because of their poor flavour, cannot be consumed directly from the vine; they are mainly processed to pickled green tomatoes among other products (fried green tomatoes, cocktail sauces, tartar sauces, chutney, marmalades) (*1*,*2*). Consumption of green tomatoes

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is considered beneficial for human health mainly due to the high concentration of health promoting reagents such as chlorophyll and tomatine (*3*).

Production of pickled tomatoes involves immersion of the fruit in NaCl solution (brining) at concentrations depending on the desired microbial control and brine stock quality (*4*). Immediately after brining, two simultaneous mass transfer fluxes are enhanced: water flows from the product to the surrounding solution and the solutes infuse into the product (*5*). Under these conditions, the semipermeable nature of the fruit cell membranes allows leakage of solutes such as minerals, vitamins and sugars from the tissue, which might be important for the quality of the final product (*5,6*). The mass transfer rate depends on the temperature of the solution, nature and molecular mass of the applied agent, the concentration of the solution as well as the geometry and cellular structure of the tissue (*7*). Additionally, the quality characteristics of the final product depend on the cultivar, variety, ripeness degree and tissue microstructure, like membrane permeability and cell porosity (*8*). In systems involving microbial fermentation, the equilibration rate of fermentable substrates and sodium chloride may affect the fermentation rate (*9*).

Apart from the solute exchange between the tissue and the surrounding solution, tissue responds to oxygen deficiency through succinate and/or alanine accumulation, glycolysis enhancement and decrement of intracellular pH and ATP levels (*10*). The alcoholic fermentation metabolism in tissue is induced to regenerate NAD^+ from the glycolytic intermediate pyruvate catalyzed by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), forming acetaldehyde and ethanol, respectively (*11*). The formation of lactate is catalyzed by lactate dehydrogenase (LDH) (*10*). Plant tissue survival under anaerobic conditions partly depends on the regeneration of NAD^+ for the production of ATP through substrate-level phosphorylation (*12*). Exposure of tomato (*13*), avocado (*10*), bell pepper (*14*) and freshly cut carrots (*15*) to anaerobic environments enhanced the activity of the enzymes involved in ethanol fermentation. High rates of fermentative metabolism may result in the accumulation of end products to deleterious levels for the commodity. Additionally, high concentrations of volatiles, such as acetaldehyde, may cause off-flavours and reduce the quality (*16*).

While the fruit fermentative metabolism has been investigated thoroughly during storage or in microbial fermentation systems, there is virtually no information on the fermentative metabolism of the fruit tissue *per se* when it is subjected to storage in brine. Therefore, the aim of this work is to investigate the effects of salt mass fraction in brine on gene expression and activity of the major plant fermentation enzymes (ADH, PDC and LDH) and the development of physicochemical characteristics of mature green tomato fruit during storage in brine.

Material and Methods

Plant material and treatments

Tomato fruit (*Solanum lycopersicum* cv. Ailsa Craig) was harvested at the mature green stage from an experimental greenhouse of the Mediterranean Agronomic Institute of Chania, Greece, and selected for uniformity of size (4–5 cm in diameter, 30–25 g) and shape before use. To prevent microbial infection, the fruits were directly washed with 20 % household bleach (6 % sodium hypochlorite), rinsed several times with sterile water and air-dried in a laminar hood. All experiments were performed under sterile conditions in a laminar hood. To study the response of fruit fermentation metabolism under different salt mass fractions, whole tomato fruits were placed in 4-litre sterile glass jars, containing 1:1 (by mass per volume) fruit and sterilized distilled water, and brined with 0 (water), 5 and 10 % NaCl. Three jars were used for each treatment, and each jar was supplemented with up to 30 fruits. Six fruits and the brine of equal volume to the fruit mass were sampled at 0, 0.5, 1, 1.5, 2, 3, 7 and 14 days of storage. All experiments were conducted at room temperature and each was repeated three times.

After the removal of the fruit from the jar, pericarp tissues including the skin of three fruits were frozen in liquid nitrogen and stored at –80 °C until the analysis for gene expression and activity, and another three were used directly for the determination of physicochemical characteristics.

Physicochemical characteristics of the fruit

Firmness of each tomato was assessed as the force needed to puncture the skin at two different, opposite halves in an equatorial area on each tomato using a penetrometer (Bishop, FT 011, Heathfield Sussex, UK) fitted with an 8-mm probe.

Following firmness measurement, the fruits were sliced, the placenta with seeds was removed and the pericarp tissue was homogenized in a blender (Diax 900, Heidolph, Kelheim, Germany). Fruit pH was measured using a digital pH meter (model Jenway 3310, Bibby Scientific L, Stone, Staffordshire, UK), soluble solid content (SSC) was determined using a digital refractometer (Palette PR-100, Atago, Tokyo, Japan) and titratable acidity (TA) was determined by titrating 5 g of fruit juice in 100 mL of double distilled H_2O, with 0.1 M NaOH to pH=8.2 as also indicated by phenolphthalein and expressed as g per 100 g of citric acid.

Enzyme assays

Fermentation enzymes were extracted and assayed according to Ke *et al.* (*11*), following the modifications of Imahori *et al.* (*13*). Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were assayed as described by Ke *et al.* (*11*) and lactate dehydrogenase (LDH) as described by Ke *et al.* (*10*). The protein content of the extracts was determined using the Bradford assay (*17*), with bovine serum albumin (BSA) as a standard. Three fruits were used for each extraction, and three independent extractions and assays were made for each storage duration. Quantification of enzyme activity was performed by monitoring the oxidation of NADH at 340 nm using a spectrophotometer.

Total RNA isolation and Northern blot

Total RNA was extracted from the fruit, according to the method of Bachem *et al.* (*18*). A total of 1 μg of

RNA was reverse-transcribed into the first strand cDNA using SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). cDNA synthesis was performed according to the manufacturer's instructions, using Oligo $dT_{12–18}$ primer. The removal of RNA complementary to cDNA was performed by incubating the samples at 37 °C for 20 min with 1 μL (2 units) of *E. coli* RNase H (Stratagene, La Jolla, CA, USA).

Degenerate and specific primers were used to amplify *adh2*, *pdc* and *ldh1* mRNAs from tomato fruit. The primers used were: $ADH2_F$: 5′–GTT GCT AAG ATT AA (CT) CCT–3′ and $ADH2_R$: 5′–TTA CC(AG) AA(AG) AA (AGT) GTA CCC TT–3′; PDC_F: 5′-CTT ATT GCC AT(CT) GCT GGT GCT TAT–3′ and PDC_R: 5′–GTA (GT)GC ATC AGC TGA (CT)TC CAC GAT–3′; $LDH1_F$: 5′-GTG CA(AG) A(AG)(CT) CAG TTG GG(AG) ATT-3′ and $LDH1_R$: 5′-TAG CAC CAC GGA GCA CAC TA-3′.

The amplified PCR products for ADH2, PDC and LDH1 were 469, 601 and 209 bp long, respectively. The amplicons were cloned into pGEM®-T Easy Vector (Promega, Madison, WI, USA) and fully sequenced using a BigDye® v. 3.0 dideoxyterminator sequencing kit (PE Biosystems, Les Ulis, France) with M13 universal primers, in an ABI Prism™ 310 genetic analyzer (PE Applied Biosystems, PerkinElmer, Norwalk, CT, USA).

Northern blotting was performed according to the Molecular Cloning Manual (*19*). The RNA was transferred to a nylon membrane (Hybond-N, Amersham Pharmachia Biotech, Little Chalfont, Buckinghamshire, UK) by capillary elution. The 20× SSPE (3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.02 M disodium EDTA·$2H_2O$ [pH=7.4]) solution was used as a transfer buffer.

The prehybridization solution consisted of 5× SSPE, 5× Denhart's reagent, 0.1 % SDS, 100 μg/μL sonicated salmon sperm DNA and 60 % formamide. The prehybridization was carried out at 42 °C overnight.

The membrane was hybridized with α32P dATP--labelled fragments of the three cDNAs. The probes were labelled by random priming using Klenow DNA polymerase as described by Sambrook and Russel (*19*). Following the hybridization, the membranes were repeatedly washed to remove any nonspecifically hybridizing DNA. All washes were done in a shaker, each wash taking 20 min. The first wash was done at room temperature and the second and third washes were warmed to 55 ºC. The washing solution consisted of 0.2× SSPE and 0.1 % SDS. After washing, the membranes were exposed to a Fuji X-ray film (Fuji Photo Film Co, Ltd, Kanagawa, Japan) at 80 °C, using an intensifying screen.

Statistical analysis

The experimental design was a completely randomized one with three replications. Data analysis was done by an analysis of variance, using the statistical software SPSS for Windows (v. 10.0.1) and mean separation was conducted by least significant difference (LSD) at 0.05 level.

Results

Changes in brine and fruit physicochemical characteristics

Brine pH for all treatments was reduced from the initial value (pH=5.28) by about one unit within the first 3 days of storage (Fig. 1a). The pH of the brine with 0 (water used as control) or 5 % NaCl brine showed a faster decrease than that with 10 % NaCl. After day 3 of storage, the pH of the control brine increased by 0.77 units (until day 7) and remained unchanged thereafter. The final pH values of brines with 5 and 10 % NaCl were pH=3.96 and pH=4.28, respectively, by day 14. No significant changes were observed in the TA of the brines of all treatments during the first 3 days of the experiment. By day 14 of storage, TA of the control brine increased to 0.107 g per 100 g, in contrast to those with 5 and 10 % NaCl, which remained considerably lower (0.024 and 0.048 g per 100 g, respectively) (Fig. 1b). Soluble solid content (SSC) of the control brine (0 % NaCl) remained unchanged throughout the 14 days of the experiment (Fig. 1c), while those of 5 and 10 % NaCl showed a decline from initial values during storage.

Fruit pH at harvest was pH=4.4 (Fig. 2a). pH of the fruit held in control brine showed an increase of 0.5 units by day two and decreased afterwards to the initial value. The pH of the fruit held in 5 and 10 % NaCl fluctuated within the first 2.5 days, then reached pH=4.3 and pH=4.5 by day 14, respectively. An increase of TA of the control fruit was observed only on day 14, when the fruits were fully deteriorated. TA fluctuated in the fruit held in brine until day 3 and then turned to the initial levels by the end of the experiment (Fig. 2b).

Initial SSC of mature green tomato fruit was 4.97 % (Fig. 2c). Fruit SSC fluctuated about 5 % for the first 3 days in all treatments. A decline of fruit SSC to as low as 2.63 % was observed in fruit kept in control brine on day 7. In contrast, fruit held in 5 and 10 % NaCl showed an increase in SSC to as high as to 6.93 and 7.53 %, respectively, until day 7.

Harvested fruit had firmness of 28.4 N. Fruit kept in 5 and 10 % NaCl followed the same pattern of firmness, having an initial decline within the first 2 days and then it remained stable. Firmness of water-treated fruit increased within the first 1.5 days to 31.9 N, and then gradually decreased to 5 N, which was considerably lower compared to the NaCl-treated ones, which were 18.1 and 11.2 N for 5 and 10 % NaCl, respectively (Fig. 3). Changes in firmness of the control fruit were greatly affected by cracking, which appeared by day 3 of the experiment. None of the salt-treated fruit had symptoms of cracking. In contrast, the formation of hollow cavities between the seed gel area and the pericarp of fruit treated with 10 % NaCl indicated the effect of dehydration.

Changes in fermentative gene expression

Fruit held in water had the highest *adh2* expression detected as early as day 0.5. There was no change observed in *adh2* by day 2, which was reduced to considerably low expression levels thereafter. No expression was detected by day 7. Fruit held in 5 % NaCl had a

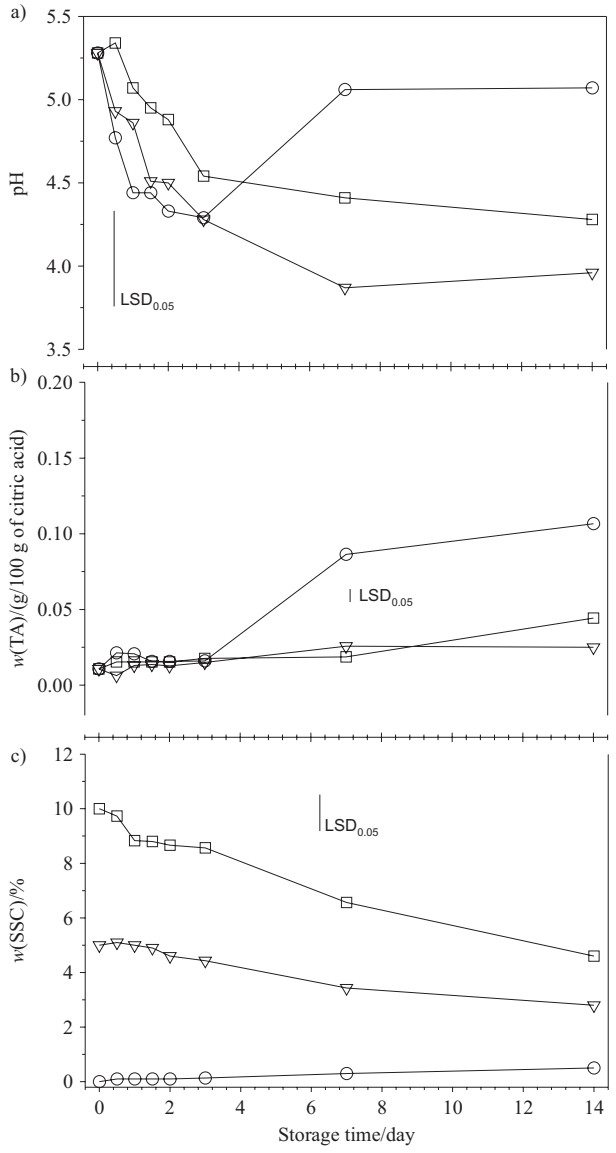

Fig. 1. Changes in: (a) pH, (b) TA and (c) SSC of brine containing 0 (water), 5 and 10 % NaCl during storage of mature green tomatoes for 14 days
○ control, ▽ 5 % NaCl, □ 10 % NaCl

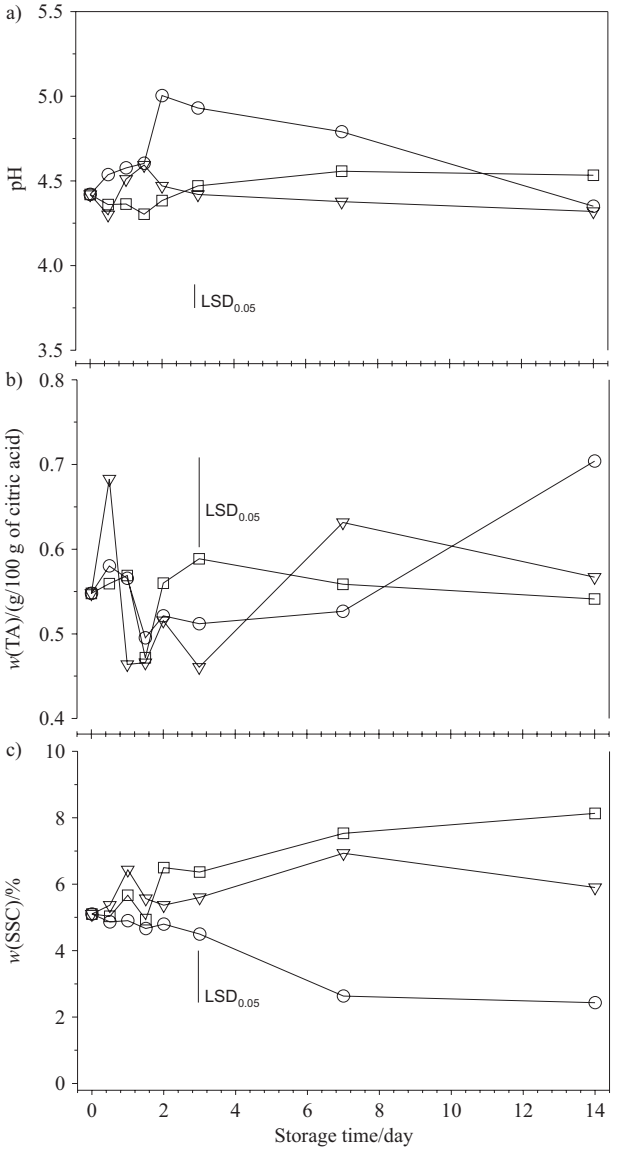

Fig. 2. Changes in: (a) pH, (b) TA and (c) SSC of tomato fruit during storage in 0 (water), 5 and 10 % NaCl for 14 days
○ control, ▽ 5 % NaCl, □ 10 % NaCl

lower expression of alcohol dehydrogenase than those held in water. However, *adh2* mRNA increased gradually in the fruit stored in 5 % NaCl, showing the highest expression until day 2 and then declined to very low levels until day 7. The induction of *adh2* mRNA was favoured more by 10 % NaCl, but unlike 5 % NaCl no expression was detected on day 7 (Fig. 4).

No *pdc* mRNA was detected in the control fruit. The pattern of *pdc* expression followed the respective treatments similarly to *adh*, however, at lower levels. Thus, the expression was higher in fruit held in water, with an expression maximum by day one of the experiment, while by day 3 only low levels of mRNA were present. Expression of *pdc* for fruit brined in 5 % NaCl was higher by day 2 and remained unchanged until day 3 of the experiment. Similarly to *adh*, *pdc* was also expressed by day 7 (Fig. 4). The initial levels of *pdc* mRNA of fruit

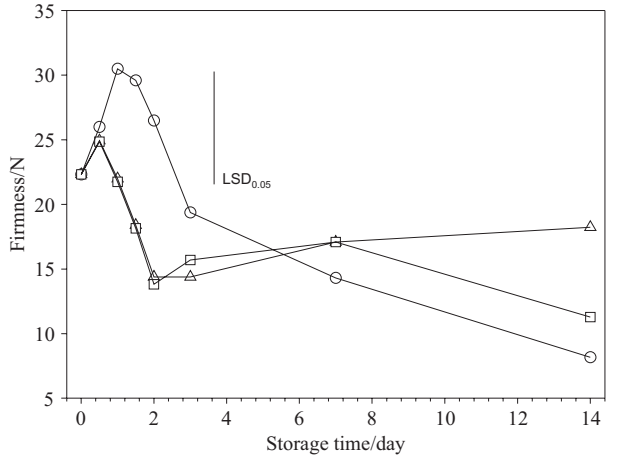

Fig. 3. Firmness of tomato fruit during storage in 0 (water), 5 and 10 % NaCl for 14 days
○ control, ▽ 5 % NaCl, □ 10 % NaCl

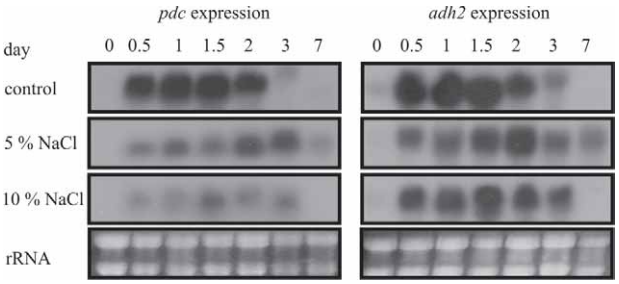

Fig. 4. Expression of *pdc* and *adh2* of mature green tomato fruit during storage in 0 (water), 5 and 10 % NaCl for 7 days

held in 10 % NaCl were lower than those held in 5 % NaCl and peaked by day 1.5. It was not possible to extract RNA from the stored fruit beyond day 14.

No *ldh*1 mRNA was detected in harvested mature green control or treated fruit (data not shown).

Changes in fermentative enzyme activities

The activity of the major enzymes (PDC, ADH, LDH) of fruit fermentative metabolism was studied in the experiment (Fig. 5). It was expressed as U per mg of protein·min. The enzymes of ethanol fermentation (PDC, ADH) had similar activities, but the activity of LDH was 5–10 times lower. PDC, ADH and LDH activities in mature green tomato before putting them in brine (0 day) were 0.62, 0.83 and 0.11 U per mg of protein·min, respectively. Water treatment stimulated PDC activity until day 7. PDC was indifferent in fruit held in 5 % NaCl brine, while in that held in 10 % NaCl, it showed an increase on day 1.5, and remained stable until day 7, when it declined to 0.05 U per mg of protein·min (Fig. 5a).

Fruit held in water showed a peak in the ADH activity on day 1.5 and a second one on day 7 to about 1.5 U per mg of protein·min. Fruit stored in 5 % NaCl showed increased activity on day 0.5, then gradually decreased to similar levels to the fruit held in water. ADH activity of fruit held in 10 % NaCl fluctuated until day 3, then decreased slowly to initial levels. By day 14 of the experiment, ADH activity was close to the initial level of the harvested fruit in all treatments (Fig. 5b).

LDH activity decreased by day 3 in fruit held in H_2O or 5 % NaCl, then it increased and peaked by day 7 in fruit held in H_2O, while in fruit held in 5 or 10 % NaCl it continued to decrease to very low levels (Fig. 5c).

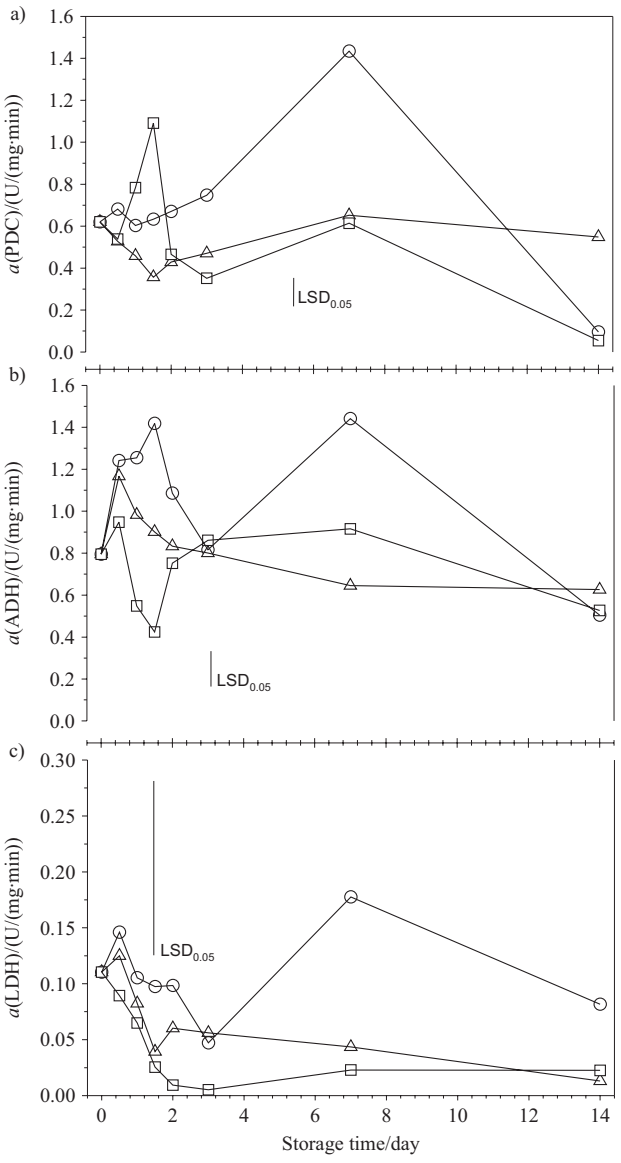

Fig. 5. Activity (*a*) in U per mg of protein·min of: (a) PDC, (b) ADH and (c) LDH of mature green tomato fruit during storage in 0 (water), 5 and 10 % NaCl for 14 days
○ control, ▽ 5 % NaCl, □ 10 % NaCl

Discussion

The aim of this study was to investigate the effect of brine strength on gene expression and activity of the major enzymes of fermentative metabolism in mature green tomato fruit. Thus, although storage in brine may last for several months, the experiments were limited to 14 days since we were unable to extract proteins from the fruit brined for longer periods (up to 30 days). Our results show that gene expression and activity of the fermentation enzymes are affected by brine strength.

During all experiments, special care was taken to avoid microbial growth that could interefere with the anaerobic response of the fruit. It is unlikely that any microbial contamination was involved in the fermentation metabolism of the tissue, since from our data we did not observe any decrease in SSC or any increase in pH or TA in neither 5 nor 10 % brining. The decrease in SSC of the control fruit was concomitant with the increase of SSC in the brine, indicating that there was a massive movement of SSC from the fruit to the surrounding solution.

Differences in osmotic potential between the fruit and the surrounding solution (brine) lead to the generation of two different types of mass transfer flows simultaneously: water flow from the product to the solution, and soluble solid (NaCl) infusion from the solution to the tissue (*5*). In tomato, water enters the fruit either

through the cuticle or the scar of the pedicel or both (*20*). In our experiment, fruit held in water cracked by day 3 of storage, as a result of the osmotic inflow of water as well as a reduction in firmness.

As a result of cracking of fruit, solutes entered the water solution altering its pH, TA and SSC. The higher TA and lower SSC, as well as the higher gene expression and activity of the enzymes of ethanol fermentative pathway, clearly show that anaerobic metabolism was induced more in fruit held in water than in fruit held in brine. An initial increase in the pH of the fruit was probably the result of higher pH of the H_2O (control brine) which entered the fruit following cracking compared to the more acidic pH of the fruit.

No cracking was observed in fruit brined in isotonic (5 % NaCl) or hypertonic (10 % NaCl) solution, since the presence of salt in the solution reduced the cracking potential. In contrast, brining of fruit in the hypertonic medium (10 % NaCl) resulted in the fruit with signs of dehydration, such as formation of hollowed cavities between the seed gel area and the outer wall. The absence of such an effect in fruit stored in 5 % NaCl suggested that the latter brine strength offers a better osmotic balance between the fruit and the surrounding solution.

Fruit held in salt solution had lower gene expression and enzyme activity. We were unable to extract RNA from the fruit treated for 14 days (or longer). A faint band indicating low gene expression was detected only in fruit brined in 5 % NaCl, whereas no expression was observed in control or 10 % NaCl fruit, showing that fruit survive longer in 5 % NaCl brine. In support of this, no significant changes in enzyme activity were observed during storage in 5 % NaCl, indicating that the lower induction of fermentative metabolism enhances the delay of deterioration and cell death.

Apart from the energy production that can be achieved by a high fermentation rate, the fermentative metabolism might result in the accumulation of acetaldehyde, ethanol and lactate by the actions of the enzymes PDC, ADH and LDH (*16*). The increase of these enzymes was found in avocado (*10*) and bell pepper (*21*) in response to low O_2. In contrast, under anoxic conditions, the activities of PDC, ADH and LDH do not change, but the corresponding end-products accumulate at high levels (*13*). Similarly, in our study, the activities of these enzymes did not change significantly (Fig. 5). Considering the fact that immersion in solution completely restricts the oxygen supply of tissue, it is possible that high production of acetaldehyde and ethanol occurred. The large increases in concentrations of fermentation volatiles in anaerobic atmospheres are detrimental to the commodity, causing the accumulation of off-flavours. Thus, the extensive deterioration that was observed in the fruit treated in water may be the result of the accumulation of those substances, which might have been concentrated at deleterious levels for the tissue.

Previous studies have shown that *ldh1* mRNA is highly expressed 3 h after hypoxic incubation of tomato fruit of breaker stage and reduced to basal levels after 18 h (*22*). No *ldh1* mRNA was detected in any of the treatments or in the control fruit, showing that lactate dehydrogenase might be expressed earlier than 12 h, which was the first time point of sampling after the start of the experiment.

Conclusions

The deterioration of fruit metabolism under anaerobic conditions is accelerated because of limited energy production, which is not enough to maintain the tissue at an acceptable quality level. Storage in brine with 0 % NaCl (water) maximised the osmotic forces that led to deterioration of fruit physicochemical characteristics (due to cracking). It also enhanced the fermentative metabolism, which might be beneficial for the tissue in terms of energy production but can also be detrimental concerning the accumulation of products of fermentative metabolism. Fruit stored in brine with 10 % NaCl only partly maintained the physicochemical characteristics; it showed signs of dehydration and its fermentative metabolism was reduced. In this study, we showed that green tomato fruit brined in 5 % NaCl maintained its physicochemical characteristics for a short period of 14 days and the duration of its fermentative metabolism was extended, though at low levels.

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