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Treatment with low-concentration acidic electrolysed water combined with mild heat to sanitise fresh organic broccoli (*Brassica oleracea*)

Qin Liu [a, b], Chih Shen Cedric Tan [c], Hongshun Yang [a, b, *], Shifei Wang [d]

[a] Food Science and Technology Programme, c/o Department of Chemistry, National University of Singapore, Singapore, 117543, Singapore
[b] National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Suzhou, Jiangsu 215123, PR China
[c] Science Research Programme, Temasek Junior College, Singapore, 469278, Singapore
[d] Changzhou Qihui Management & Consulting Co., Ltd, Changzhou, Jiangsu 213000, PR China

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ABSTRACT

The effects of low-concentration electrolysed water (LcEW) (4 mg/L free available chlorine) combined with mild heat on the safety and quality of fresh organic broccoli (*Brassica oleracea*) were evaluated. Treatment with LcEW combined with mild heat (50 °C) achieved the highest reduction in naturally occurring microorganisms and pathogens, including inoculated *Escherichia coli* O157:H7 and *Listeria monocytogenes* ($P < 0.05$). In terms of the antioxidant content of the treated broccoli, the total phenolic levels and ferric reducing antioxidant power remained unchanged; however, the oxygen radical absorbance capacity of the treated broccoli was higher than that of the untreated control. In addition, mild heat treatment resulted in an increase in firmness. The increased firmness was attributed to changes in the pectin structure, including the assembly and dynamics of pectin. The results revealed that mild heat induced an antiparallel orientation and spontaneous aggregation of the pectin chains. This study demonstrated that LcEW combined with mild heat treatment was effective to reduce microbial counts on fresh organic broccoli without compromising the product quality.

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1. Introduction

The organic food market has grown continuously over recent years worldwide (Li et al., 2015), mainly due to the low level of pesticide residues (Yu & Yang, 2017). Organic agriculture standards prohibit the use of chemosynthetic pesticides and fertilisers; however, they recommend regular composting of manure into soils (Leifert, Ball, Volakakis, & Cooper, 2008; Yang, Dey, Buchanan, & Biswas, 2014). Thus, organic fresh vegetables can represent a source of foodborne infections from this use of manure, which represent an increased risk to public health. Outbreaks of *Escherichia coli* O157:H7 infections linked to organic produce have resulted in increased levels of concern for the safety of such food material; meanwhile, *Listeria monocytogenes* is an emerging pathogen for fresh produce, therefore necessitating the development of sanitising methods for organic produce (Centers for Disease Control and Prevention, 2012).

Strict requirements apply to the production and processing of organic products. The National Organic Program (NOP) provides clarification regarding the use of chlorine materials in organic production and handling, dictating that residual chlorine levels must not exceed the maximum residual disinfectant limit of 4 mg/L under the drinking water act (NOP, 2011). Sodium hypochlorite is one of the most commonly used agents to reduce the microbial contamination of freshly cut vegetables. However, the main disadvantage of using traditional chlorine-based sanitisers is the possible formation of harmful by-products, such as chloramines and trihalomethanes, which have led to concerns regarding their use in the fresh-cut vegetable industry (Singh, Singh, Bhunia, & Stroshine, 2002). Therefore, the development of alternative sanitising methods to reduce microbial contamination, which are compatible with regulations related to the processing of fresh organic produce, is essential.

The use of low concentration electrolysed water (LcEW), which possesses several potential advantages over traditional chlorine-

* Corresponding author. Food Science and Technology Programme, c/o Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore.
E-mail address: chmynghs@nus.edu.sg (H. Yang).

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based sanitisers, represents a promising alternative (Ding et al., 2015; Huang, Hung, Hsu, Huang, & Hwang, 2008; Yang, Feirtag, & Diez-Gonzalez, 2013; Zhang & Yang, 2017; Zhao, Zhang, & Yang, 2017). The bactericidal effect of LcEW is partially due to its high oxidation potential (ORP), which could cause the modification of metabolite fluxes and electron flow in cells (Huang et al., 2008). LcEW containing 2–5 mg/L of residual chlorine has an ORP range of 941–1010 mV, which is outside the preferable redox potential range of most microorganisms (Park, Hung, & Chung, 2004). LcEW has been reported to act as an effective bactericidal agent and may represent a suitable sanitiser for organic produce, as it does not affect the quality of the produce and is safe for use (Koseki, Yoshida, Kamitani, Isobe, & Itoh, 2004; Rahman, Ding, & Oh, 2010a, 2010b). Thus, LcEW might replace the widely used strong acidic EW, especially for organic produce.

Moderate heat shock is commonly used to treat vegetables (Kim, Nimitkeatkai, Choi, & Cheong, 2011). Thus, a combination of LcEW and mild heat might be effective and show synergistic effects. The use of food sanitisers in combination with additional methods, with the aim of achieving improved food safety without loss of quality, is of great interest to the research community.

Broccoli (*Brassica oleracea*) is highly sought after because of its nutritional value and antioxidant activities. EW-based treatment of microbial contamination of broccoli showed promising results (Hung, Tilly, & Kim, 2010); however, the impact of EW-based treatment on quality has received little attention to date. In addition, texture is one of the most important sensory characteristics of edible vegetables.

The objective of this work was to investigate the antimicrobial efficiency of treatment with LcEW (4 mg/L) combined with mild heat and to evaluate the effect of this treatment on nutrient quality, texture, and cell wall changes of organic broccoli.

2. Materials and methods

2.1. Materials

Certified organic broccoli was purchased from a local supermarket in Singapore. The broccoli were packed in a polyethylene bag and stored at 4 °C and used for the experiments within 24 h. Samples were selected that were similar in size and colour, with no visible yellowing or damage symptoms. Each single set comprised approximately 10 g of broccoli and triplicate samples from each treatment were taken for analysis.

2.2. Bacterial strains and inoculation

Strains of *E. coli* O157:H7 (C7927) and *L. monocytogenes* (ATCC BAA-839) were obtained from Dr. Yuk Hyun-Gyun, Food Science and Technology Programme, National University of Singapore. *E. coli* O157:H7 and *L. monocytogenes* were adapted to 100 mg/mL nalidixic acid (Sigma-Aldrich, USA) by stepwise increment after each transfer of the respective culture. All media used in this study were supplemented with 100 mg/mL nalidixic acid so that these pathogens isolated from broccoli were relatively free from other background bacterial contaminants. Inoculation suspensions of *E. coli* O157:H7 and *L. monocytogenes* were prepared by diluting each culture in 0.1% peptone water to achieve a final cell number of approximately 10^7 colony forming unit (CFU)/mL. Broccoli sprouts were submerged in the bacterial suspension for 5 min. The inoculated broccoli sprouts were then air-dried in a laminar flow biosafety cabinet for 30 min.

2.3. Sanitising treatments

The electrolysed water was obtained by the electrolysis of dilute sodium chloride solution using an electrolysed water generator (ROX-10WB3, Hoshizaki Electric Company, Aichi, Japan). Dilution with deionised water was used to achieve a free available chlorine (FAC) level of 4 mg/L, which was measured using a reflectometer (Merck, Darmstadt, Germany). Broccoli sprouts (10 g) were dipped into 200 mL of each treatment solution. After the dipping, samples were dried in the laminar flow cabinet for 30 min.

To determine the effectiveness against the natural microbiota of broccoli, samples were processed with LcEW (FAC, 4 mg/L) at 20, 40 and 50 °C for various holding times (3, 5, 10 min). Subsequently, the combination of LcEW with mild heat (50 °C) for 5 min was chosen to further explore the potential of the combined treatment to inactivate pathogens and the impact of the treatments on the analytical parameters.

2.4. Microbiological analysis

A 10 g of sample from each treatment was aseptically transferred into in a stomacher bag containing 90 mL of 0.1% peptone water. Samples were then homogenised using a Stomacher (Masticator Stomacher, IUL Instruments, Germany) for 180 s. Serial dilution was performed and 0.1 mL of each dilution was used for plate spreading. Pathogenic counts were determined by plating appropriately diluted samples onto tryptic soy agar (TSA, Oxoid Limited, Hampshire, UK) containing 100 mg/L nalidixic acid. Mesophilic aerobic microorganisms were determined from plate count agar (PCA, Oxoid, UK), which were incubated at 37 °C for 2 days. Yeast and mould counts were determined from potato dextrose agar (PDA, Oxoid, UK) with incubation at 25 °C for 3 days. The results were expressed as the average colony forming units per gram of fresh weight (CFU/g FW).

2.5. Total phenolic content and antioxidant capacity of organic broccoli

2.5.1. Total phenolic content

The total phenolic content of broccoli was determined via the Folin-Ciocalteu assay (Singleton, Orthofer, & Lamuela-Raventos, 1999), using gallic acid as a standard. Absorbance was measured in triplicate. Results were expressed as mg of gallic acid equivalents per 100 g of fresh weight (GAE)/100 g FW.

2.5.2. Antioxidant activity analysis

Sample extracts were prepared according to the method of Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Byrne (2006), with some modifications. The extracts were obtained from the percolation method of plant material in methanol 1:10 (plant material (g): solvent (mL)). Ten grams of broccoli floret tissue was homogenised with 100 mL of methanol. The homogenates were kept at 4 °C for 12 h and then filtered. The supernatants were recovered and stored at −20 °C in amber bottles for protection from light until analysis. The ferric reducing antioxidant power (FRAP) assay was performed according to a previous report (Benzie & Strain, 1996). A solution of $FeSO_4$ was used for calibration. Results were expressed as μmol of $FeSO_4$ equivalents/100 g FW. The oxygen radical antioxidant capacity (ORAC) assay was performed based on previous reports (Fu et al., 2015a, 2015b). Trolox (12.5–100 μM) was used as a standard. ORAC values were expressed as μM of Trolox Equivalent (TE)/g FW.

2.6. Texture and pectin content

The firmness of broccoli stem was determined as described by Christiaens et al. (2011). Textural analysis was performed using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Goldaming, Surrey, UK).

The preparation of cell walls from broccoli and the extraction of water-soluble pectin (WSP), cyclohexane-trans-1, 2-diamine tetra-acetic acid (CDTA) soluble (CSP) and sodium carbonate-soluble pectin (SSP) fractions were performed according to a previously published method (Chong, Lai, & Yang, 2015). The pectin fractions were analysed for their galacturonic acid (GalA) levels according to the carbazole method (Bitter & Muir, 1962).

2.7. Nanostructural characterisation of pectin

Samples were prepared for pectin atomic force microscope (AFM) imaging according to a previously published method (Chong et al., 2015; Yang, 2014). Characterisation of pectin nanostructure was carried out using a TT- Atomic Force Microscope (AFM workshop, Signal Hill, CA, USA) equipped with a Sensaprobe TM190-A-15 tip (Applied Nanostructures, Mountain View, CA, USA). Gwyddion software was used to process the AFM images.

2.8. Statistical analysis

Mean values for all parameters were calculated from the independent triplicate trials. The results were analysed using one-way analysis of variance (ANOVA) in the statistical software SPSS v18 (Statistical Package for the Social Sciences, Chicago, IL, USA). Differences between means were subject to Duncan's test and a difference at $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Efficacy of combined treatment with electrolysed water and heat against native microflora in organic broccoli

The effects of dipping temperatures (20, 40, and 50 °C) on the efficiency of EW against aerobic mesophilic counts (AMC), yeasts, and moulds present in broccoli are shown in Fig. 1A. For AMC, washing with EW at 40 °C for 5 min resulted in a significantly greater ($P < 0.05$) sanitising effect (1.77 log CFU/g FW reduction) than treatment with deionised water (DI) (1.12 log CFU/g FW reduction) relative to the untreated control. However, the sanitising effect of EW at 50 °C (2.20 log CFU/g FW reduction) was much stronger than that of DI (1.3 log CFU/g FW reduction). As the dipping temperature increased from 20 to 50 °C, both DI- and EW-based treatments resulted in significant reductions in microorganisms.

Similar results were observed for each treatment in terms of reduction in the numbers of moulds and yeasts. However, a significant difference was observed in terms of the reduction of numbers following treatment with DI and EW at 20, 40 and 50 °C. Treatment with EW resulted in a significantly greater ($P < 0.05$) sanitising effect (2.32 log CFU/g FW reduction) than treatment with DI (1.13 log CFU/g FW reduction). The application of mild heat was reported to enhance the efficacy of sanitising agents in terms of the inactivation of microorganisms present in product. Taormina and Beuchat (2002) reported that bactericidal effects resulting from treatment with 1.5 g/L calcinated calcium at 50 °C were stronger than those obtained by washing iceberg lettuce with distilled water at 45 °C. Rahman, Jin, and Oh (2010) reported that an increase in dipping temperature from 20 to 50 °C resulted in a significantly larger reduction in total bacteria (from 2.70 to 3.98 log CFU/g FW) and yeasts and moulds (2.69–3.45 log CFU/g FW) when combined with treatment using 1% citric acid and washing with alkaline EW ($P < 0.05$).

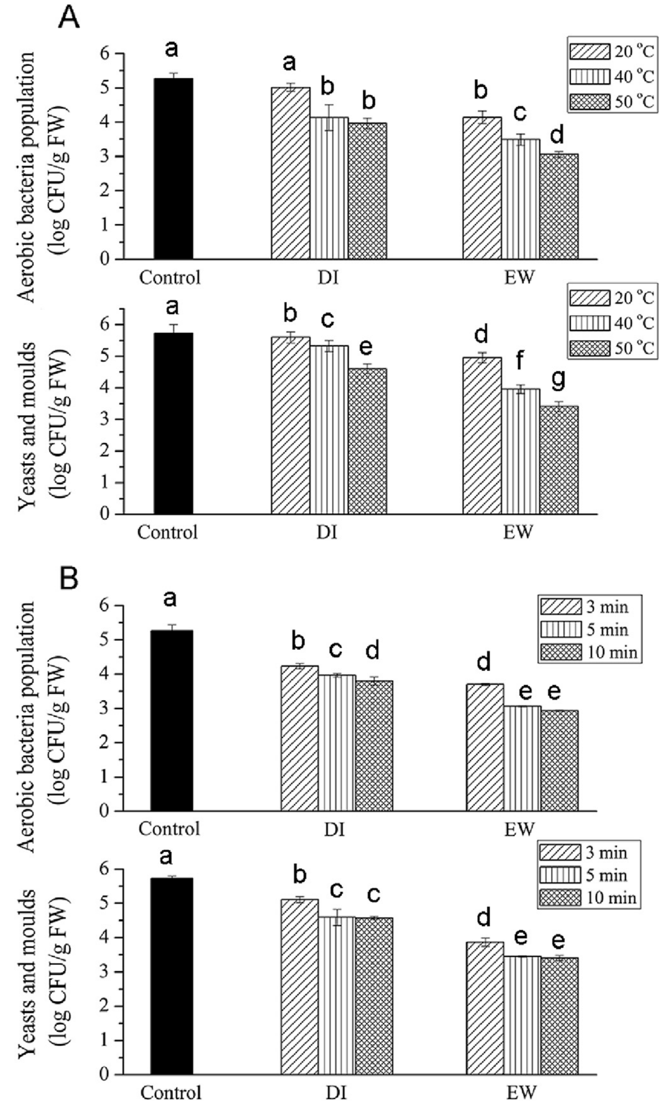

Fig. 1. Effect of treatment with electrolysed water on the inactivation of aerobic mesophilic bacteria, yeasts and moulds on organic broccoli at different dipping temperatures (A) and different dipping durations (B); Mean values indicated by different letters in the same figure are significantly different ($P < 0.05$); (DI: deionised water; EW: electrolysed water with 4 mg/L of free available chlorine).

The effect of duration of dipping (3, 5 and 10 min), in combination with treatment with the sanitising agent, on the reduction of AMC, yeasts, and moulds present in organic broccoli, is shown in Fig. 1B. Treatment with EW for 10 min resulted in a greater reduction in AMC, yeasts, and moulds than that observed in the group treated with DI (0.85 and 1.17 log CFU/g FW, respectively). No significant difference ($P > 0.05$) was found on dipping in EW for 5 min and 10 min; however, in both groups, the reductions of AMC, yeasts, and moulds were greater than those observed on dipping for 3 min ($P < 0.05$). Therefore, a 5-min exposure was considered suitable to achieve the desired reduction in microbe numbers in organic broccoli.

3.2. Sanitising effect on E. coli O157:H7 and L. monocytogenes

Treatment of inoculated broccoli sprouts with EW resulted in a significant reduction in the population of *E. coli* O157:H7 and *L. monocytogenes* (Fig. 2). The application of mild heat combined with LcEW resulted in the greatest reduction in the *E. coli* O157:H7 population (by 2.14 log CFU/g FW) compared with the untreated group, whereas treatment with DI at 50 °C resulted in a 1.16 log CFU/g FW reduction. In addition, it should be noted that treatment with mild heat at 50 °C did not achieve a significantly greater reduction in the numbers of *L. monocytogenes* compared with treatment with LcEW alone; however, treatment with LcEW at 50 °C resulted in a 2.12 log CFU/g FW reduction of *L. monocytogenes* compared with that of the untreated group (6.77 log CFU/g FW).

Our results indicated that treatment with EW containing 4 mg/L free available chlorine (FAC) was significantly more effective ($P < 0.05$) than treatment with DI, at 20 or 50 °C, in terms of reducing the population of *E. coli* O157:H7 and *L. monocytogenes* in organic broccoli. An increase in temperature (from 20 to 50 °C) did not result in a significant ($P > 0.05$) reduction in the populations of *E. coli* O157:H7 and *L. monocytogenes*.

3.3. Total phenolic content and antioxidant capacity of organic broccoli

Changes in total phenolic content and antioxidant activity of organic broccoli are shown in Table 1. Following treatment with mild heat, the phenolic content of the DI-treated samples decreased to 169.3 ± 0.4 mg GAE/g of broccoli. Treatment with EW at 50 °C resulted in a significantly higher ($P < 0.05$) total phenolic content than treatment with DI. EW-treated samples were found to have a 22.9% higher total phenolic content than the DI- treated samples, following heat exposure at 50 °C. Sanitation using EW might act as an inducer of abiotic stress, which could result in the accumulation or enhancement of the total phenolic content; similar effects have been reported in other fresh vegetables following various sanitation methods, such as exposure to UV-C radiation (Jiang, Jahangir, Jiang, Lu, & Ying, 2010).

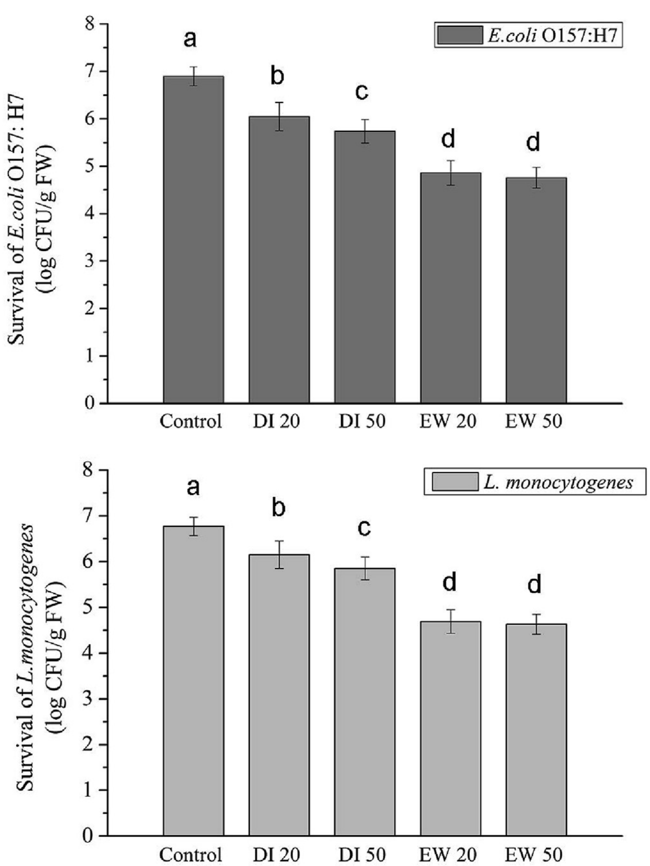

Fig. 2. Effect of treatment with electrolysed water on the inactivation of *E. coli* O157:H7 (A) and *L. monocytogenes* (B) in organic broccoli at 50 °C for 5 min; (DI 20, Deionised water at 20 °C; DI 50, Deionised water at 50 °C; EW 20, Electrolysed water at 20 °C; EW 50, Electrolysed water at 50 °C).

Table 1
Effects of different treatments on the total phenolic content and antioxidant capacity of organic broccoli.

Treatment	Total phenolic content (mg GAE/100 g FW)	Antioxidant capacity	
		ORAC (μmol eq. Trolox/100 g FW)	FRAP (μmol eq. $FeSO_4$/100 g FW)
Untreated	206.4 ± 1.2 [a]	52.1 ± 2.5 [a]	525.5 ± 17.5 [a]
DI 20	205.9 ± 0.3 [a]	52.5 ± 2.4 [a]	522.6 ± 14.3 [a]
DI 50	169.3 ± 0.4 [b]	54.0 ± 2.9 [ab]	538.5 ± 25.5 [a]
EW 20	206.0 ± 2.3 [a]	51.9 ± 4.2 [a]	516.9 ± 21.5 [a]
EW 50	208.1 ± 1.3 [a]	59.2 ± 2.4 [b]	536.7 ± 20.2 [a]

FW: fresh weight, DI 20, Deionised water at 20 °C; DI 50, Deionised water at 50 °C; EW 20, Electrolysed water at 20 °C; EW 50, Electrolysed water at 50 °C.
Data are presented as mean ± standard deviation (n = 3). Different letters indicates significance difference for mean ($P < 0.05$).

To evaluate the antioxidant activity, usually, at least two *in vitro* assays are conducted to give comparable results because of the differences between the test systems (Schlesier, Harwat, Böhm, & Bitsch, 2002). The antioxidant properties of organic broccoli, as a measure of the ORAC and FRAP values, are shown in Table 1. The ORAC value of untreated broccoli was 52.1 μmol TE/100 g of broccoli. Treatment with EW and DI did not affect the antioxidant levels (ORAC) relative to the untreated control at 20 °C. Treatment with mild heat resulted in an increase in the total ORAC of the DI- and EW-treated samples, to 54.0 and 59.2 μmol TE/100 g of broccoli, respectively. The ORAC values of the DI- and EW-treated samples increased by 3.6% and 13.6%, respectively, relative to those of the untreated control. Previous studies showed that thermal treatment might increase the nutritional value compared to that of fresh produce. Roy, Juneja, Isobe, and Tsushida (2009) found that steam processing elevated the total ORAC (hydrophilic and lipophilic) value of broccoli by 2.3-fold.

A FRAP assay was performed to assess the total non-enzymatic 'antioxidant' activity of organic broccoli. No significant differences ($P > 0.05$) in FRAP values were found after treatment with DI and EW at 20 °C. However, the application of mild heat at 50 °C affected the FRAP levels, which increased by 2.47% and 2.13% in DI- and EW-treated samples, respectively. Various studies have indicated that thermal treatment preserves the nutritional qualities and storage stability of fresh broccoli (Roy et al., 2009). No correlation was found between antioxidant values and total phenolic content, indicating that the antioxidant capacity of broccoli might be attributed to other molecules, such as ascorbic acid, carotenoids, flavonoids and glucosinolates (Hwang & Lim, 2014).

3.4. Firmness and pectin content

Fig. 3 shows the firmness of treated broccoli stems relative to that of untreated broccoli stems. An increase in firmness was observed when broccoli was treated with DI and EW for 5 min at 50 °C. The positive effect of mild heat on the texture of processed broccoli was consistent with results in other fruit and vegetables (Abreu, Beirão-da-Costa, Gonçalves, Beirão-da-Costa, & Moldão-Martins, 2003; Anthon & Barrett, 2006). The increase in firmness could reflect the enzymatic activity of pectin methylesterase (PME) in a low-temperature blanch, resulting in greater cell-cell adhesion,

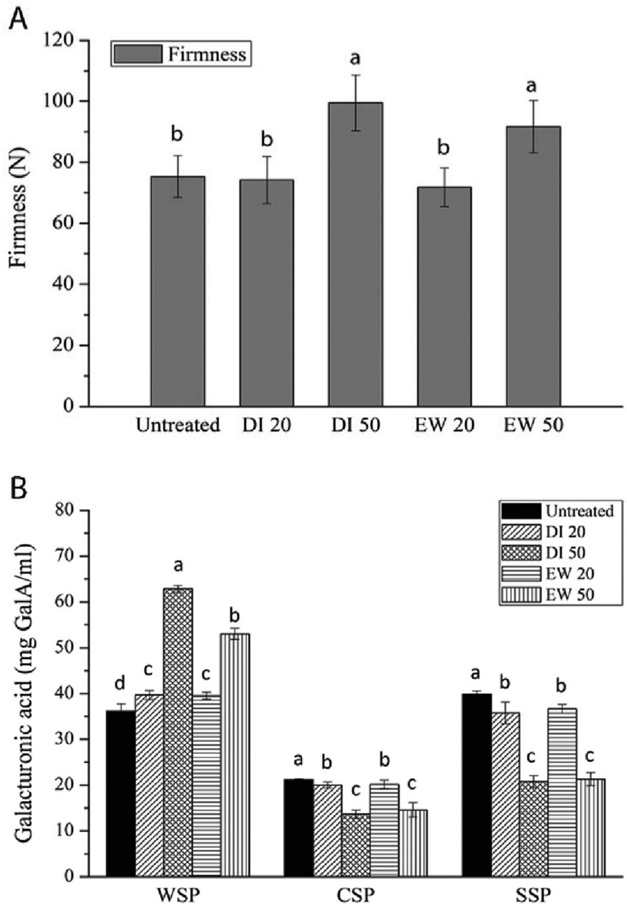

Fig. 3. Firmness (A) and the content of pectin solubilised in water (WSP), cyclohexane-trans-1,2-diamine tetra-acetic acid (CSP), and sodium carbonate (SSP) (B) in broccoli.

described as the ‘egg-box’ model, which increases the rigidity of plant tissue (Abreu et al., 2003; Ventura, Jammal, & Bianco-Peled, 2013). In view of this, treatment with mild heat is crucial for maintaining or improving the texture of broccoli.

Pectic polymers were fractionated into three main fractions, based on their differential solubility in water (WSP) as well as in cyclohexane-trans-1, 2-diamine tetra-acetic acid (CSP) and sodium carbonate (SSP). The WSP fraction contained polyuronides that were loosely bound to the cell wall via non-covalent and non-ionic bonds, whereas the CSP and SSP fractions consisted primarily of ionic and covalent ester bonds, respectively (Cybulska, Zdunek, & Kozioł, 2015). Untreated broccoli contained considerable amounts of both CSP (21.17 mg GalA/mL) and SSP (39.89 mg GalA/mL). Similar levels of GalA were found in the untreated control and EW-treated broccoli samples at 20 °C, which suggested that treatment with EW did not affect the pectin composition of the cell wall significantly. Broccoli treated with mild heat and DI or EW showed a remarkable decrease in SSP and a less marked decrease in CSP compared with the untreated group. The decrease was concomitant with an increase in the loosely bound WSP.

3.5. Nanostructure of SSP molecules

Molecular arrangement is critical to determine the roles of cell wall polysaccharides (Chen et al., 2013). Accordingly, changes in the molecular structure of pectin, at the nanostructural level, may be studied by AFM (Morris, Gromer, Kirby, Bongaerts, & Gunning, 2011).

AFM images (Fig. 4A) clearly show the branched structure of the SSP fraction of the broccoli samples. Fig. 4 (a) shows that the SSP fraction forms a self-assembled network on mica. In the untreated group, a significantly larger number of branched structures, as well as fractions with multiple branching structures, were present. The ability of SSP of broccoli to undergo self-assembly has not been reported previously. The previously reported dimensions of the SSP molecules are in general agreement with our data. Similar networks have been observed in carrot and pear (Cybulska et al., 2015; Zdunek, Kozioł, Pieczywek, & Cybulska, 2014); however, these were different in terms of branching and interlocking.

With increasing treatment temperature, the regular interlinked network was observed to disappear, and aggregate-like complexes were observed between the linear molecules. Fig. 4(d) and 4(f) show typical images of pectic materials, which were observed as an aggregated complex and were considered to consist of a main

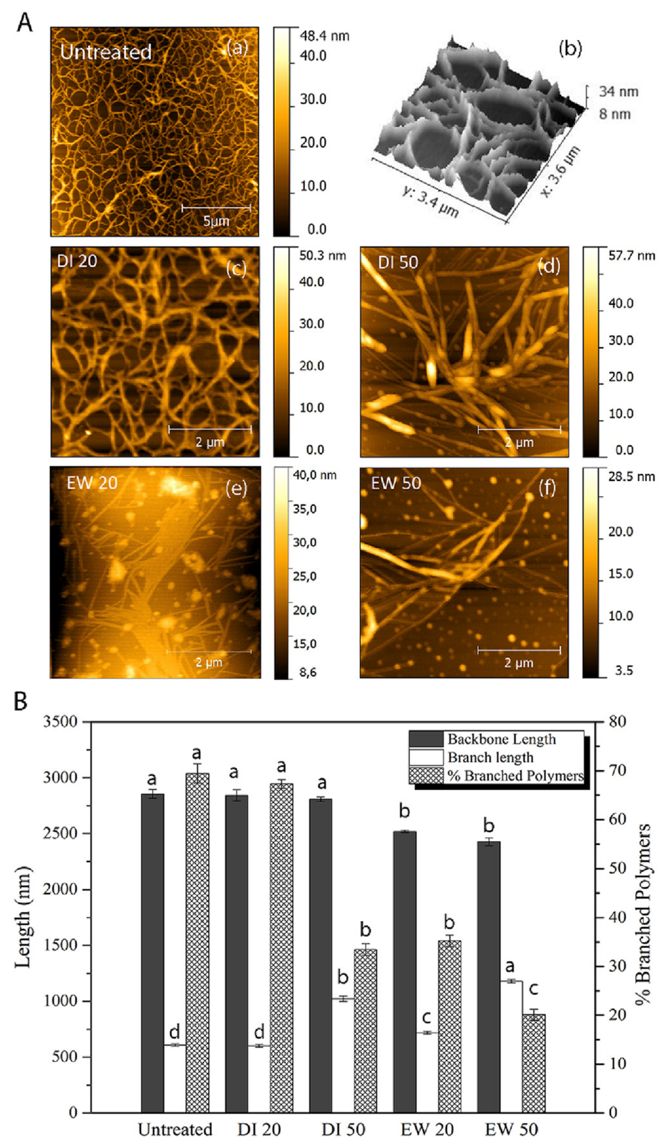

Fig. 4. Atomic force microscopy (AFM) images of sodium carbonate (SSP) chains in broccoli (A) and changes in mean backbone and branch length, and the percentage of pectin in branches (B); (a) untreated group (b) three-dimensional image of the untreated group; (c) DI 20 (d) DI 50 (e) EW 20 (f) EW 50; (DI 20, Deionised water at 20 °C; DI 50, Deionised water at 50 °C; EW 20, Electrolysed water at 20 °C; EW 50, Electrolysed water at 50 °C).

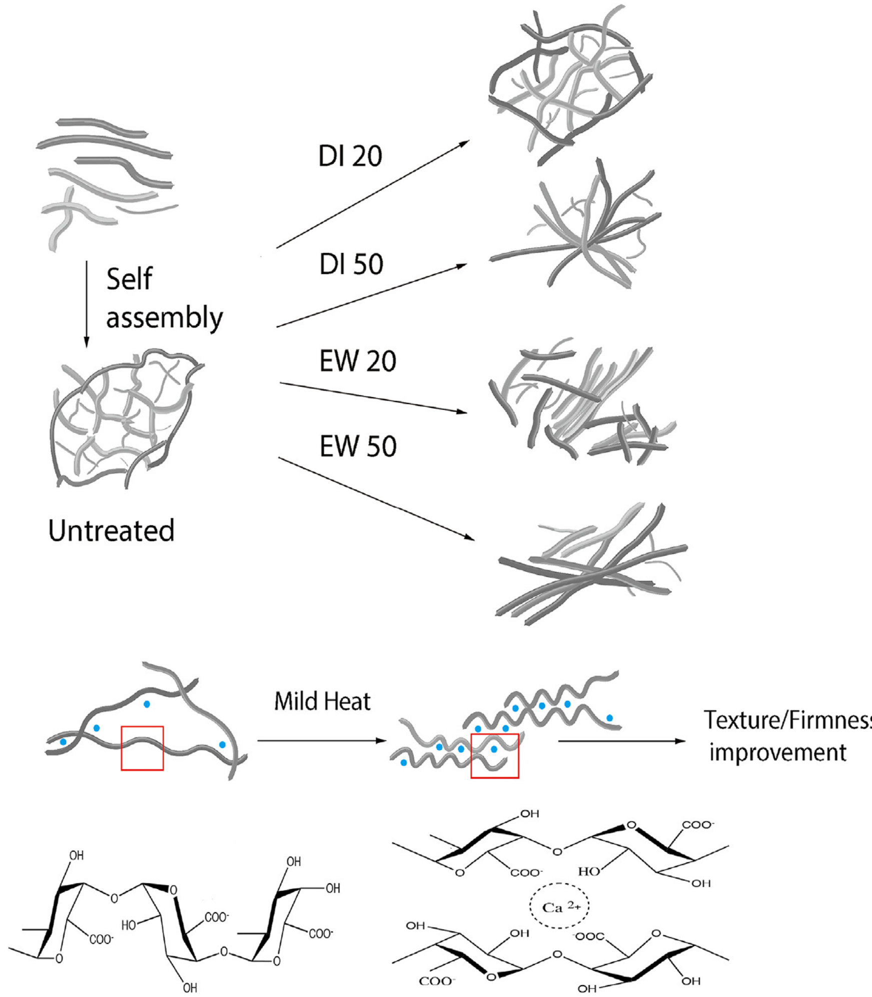

Fig. 5. Schematic illustration of the changes in the pectin nanostructure in response to various treatments.

backbone and side polysaccharides (Morris et al., 2011). The long straight chains likely represented the homogalacturonan (HG) backbone, to which the 'hairy' parts of the rhamnogalacturonan (RG) were covalently linked. The model describing the formation of these pectin junction zones, which likely occur via associations between blocks of polygalacturonic acid, is known as the 'egg-box' model (Morris et al., 2011; Ventura et al., 2013). Fig. 4B shows that treatment with EW induced pronounced changes in the structure of the SSP fraction, reflected by the shift from sparsely branched pectin to shorter and linear ones, which may be attributed to the activity of polygalacturonase (Cybulska et al., 2015). A significant reduction in the mean backbone and branch length, and the percentage of branched polymers, was observed after treatment with mild heat or EW, indicating degradation of the pectin chains and a decline in the number of branched pectin molecules. Fig. 5 shows a schematic illustration of the changes in the pectin structure of broccoli following various treatment methods. Anthon and Barrett (2006) reported that low-temperature blanching activates PME, resulting in the interaction of free carboxylic acid groups with Ca^{2+}, thereby inducing the formation of the 'egg box' structure. According to the 'egg box' model, the linking of pectin chains involves cooperative binding of calcium ions (Morris et al., 2011). Following exposure to mild heat, the antiparallel orientation of the pectin chains appeared to be the most favourable arrangement, suggesting that changes in pectin structure represent a possible mechanism underlying the increase in the firmness of vegetables following thermal treatment.

4. Conclusions

The combination of LcEW and mild heat was found to yield a synergistic improvement in the bactericidal efficacy against aerobic bacteria, yeasts and moulds present in fresh organic broccoli, as well as on the inoculated *E. coli* O157:H7 and *L. monocytogenes*. Treatment with EW (4 mg/L) combined with heat (50 °C) resulted in a greater than 2 log CFU/g FW reduction in the count of aerobic mesophilic bacteria, yeasts and moulds, as well as in the population of pathogens. The application of the combined treatment enhanced the microbial safety but did not affect the quality of the broccoli significantly. Mild heat improved the broccoli's firmness. The effect of EW and mild heat treatment on the nanostructure of pectin was determined via AFM analysis, providing an improved understanding of the disassembly process that occurs during the firming of broccoli.

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