Thermal Stability of L-Ascorbic Acid and Ascorbic Acid Oxidase in Broccoli (*Brassica oleracea var. italica*)

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ABSTRACT: The thermal stability of vitamin C (including L-ascorbic acid [L-AA] and dehydroascorbic acid [DHAA]) in crushed broccoli was evaluated in the temperature range of 30 to 90 °C whereas that of ascorbic acid oxidase (AAO) was evaluated in the temperature range of 20 to 95 °C. Thermal treatments (for 15 min) of crushed broccoli at 30 to 60 °C resulted in conversion of L-AA to DHAA whereas treatments at 70 to 90 °C retained vitamin C as L-AA. These observations indicated that enzymes (for example, AAO) could play a major role in the initial phase (that is, oxidation of L-AA to DHAA) of vitamin C degradation in broccoli. Consequently, a study to evaluate the temperature–time conditions that could result in AAO inactivation in broccoli was carried out. In this study, higher AAO activity was observed in broccoli florets than stalks. During thermal treatments for 10 min, AAO in broccoli florets and stalks was stable until around 50 °C. A 10-min thermal treatment at 80 °C almost completely inactivated AAO in broccoli. AAO inactivation followed 1st order kinetics in the temperature range of 55 to 65 °C. Based on this study, a thermal treatment above 70 °C is recommended for crushed vegetable products to prevent oxidation of L-AA to DHAA, the onset of vitamin C degradation.

Practical Application: The results reported in this study are applicable for both domestic and industrial processing of vegetables into products such as juices, soups, and purees. In this report, we have demonstrated that processing crushed broccoli in a temperature range of 30 to 60 °C could result in the conversion of L-ascorbic acid to dehydroascorbic (DHAA), a very important reaction in regard to vitamin C degradation because DHAA could be easily converted to other compounds that do not have the biological activity of vitamin C.

Keywords: ascorbic acid, ascorbic acid oxidase, broccoli, dehydroascorbic acid, thermal processing

Introduction

Vitamin C is one of the numerous phytonutrients found in broccoli and other vegetables (Vallejo and others 2002, 2003). In the past decades, vitamin C was mainly popular for its role in prevention of scurvy. However, recent epidemiological evidence has revealed that vitamin C is a potent antioxidant, which could protect the human body against chronic diseases such as cataract, cancer, and cardiovascular disease (Carr and Frei 1999; Davey and others 2000).

Vitamin C is found in high amounts in fresh fruits and vegetables where it mainly occurs as L-ascorbic acid (L-AA). Dehydroascorbic acid (DHAA), the oxidized form of L-AA, also occurs but in small concentrations (Wills and others 1984). L-AA is a labile molecule whose concentration could be decreased during domestic and industrial processing of fruits and vegetables due to the occurrence of enzymatic and nonenzymatic degradation. The extent of enzymatic and nonenzymatic degradation of L-AA is influenced by factors such as concentration, oxygen, light, pressure, temperature, metal ions, sugars, and pH (Rojas and Gershenson 1997; Davey and others 2000; Oey and others 2006). Enzymatic degradation of L-AA mainly involves oxidation to DHAA, a compound reported to exhibit antioxidant properties in addition to antiscorbutic activity equivalent to that of L-AA. However, DHAA can be rapidly and irreversibly hydrolyzed to 2,3-diketogulonoic acid (2,3-DKG) hence losing its antiscorbutic activity (Deutsch 2000).

During plant growth, ascorbic acid oxidase (AAO; EC 1.10.3.3) and ascorbic acid peroxidase (APx; EC 1.11.1.11) catalyze the oxidation of L-AA. AAO catalyses the reduction of molecular oxygen to water with simultaneous oxidation of L-AA (Pignocchi and others 2003; De Tullio and others 2007). On the other hand, APx catalyzes the reduction of hydrogen peroxide with the simultaneous oxidation of L-AA (Noctor and Foyer 1998). Although it is generally recognized that AAO and APx could oxidize L-AA during vegetable processing, neither the extent of enzyme-catalyzed L-AA oxidation nor inactivation of these enzymes has been studied in detail. In plants, AAO has been detected in the cell wall, in the extracellular matrix and in the vacuole (Chichiriccò and others 1989; Liso and others 2004) whereas L-AA has been detected in the cytosol, apoplast, cell wall, and in organelles such as chloroplasts, mitochondria, and vacuole (Davey and others 2000; Liso and others 2004). Fruit and vegetable processing treatments that result in matrix disruption (for example, peeling, crushing, and cutting) could bring L-AA into contact with AAO thus facilitating oxidation of L-AA. It is therefore necessary to optimize processing conditions to sufficiently inactivate AAO and other quality

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doi: 10.1111/j.1750-3841.2010.01573.x

THE Society for Food Science & Technology
Journal of Food Science
A Publication of the Institute of Food Technologists

degrading enzymes and ensure optimum L-AA retention. Hereto, the objective of this study was to study the thermal stability of L-AA and AAO in crushed broccoli. To the best of our knowledge, such an integrated study on L-AA and AAO stability has not been previously performed.

Materials and Methods

Broccoli sample preparation

Fresh broccoli was purchased from a local market and stored at 4 °C for not more than 5 days before use. Broccoli florets (including approximately 1 cm of stalk) were excised from several broccoli heads and thoroughly mixed to obtain a homogenous sample. Stalk samples were prepared by cutting the broccoli part left after removal of florets (excluding about 3 cm of the bottom portion) into pieces measuring approximately 1 cm, which were mixed into a homogeneous sample.

Study on thermal stability of L-AA in raw crushed broccoli

To determine the native profile of vitamin C, a portion of the florets was vacuum packed in polyethylene bags and blanched by immersion in boiling water for 10 min to inactivate enzymes. The blanched florets were immediately cooled in ice water, frozen in liquid nitrogen, and crushed into a frozen powder using Grindomix (GM 2000, Retsch, Germany) at 7500 rpm for 45 s. The frozen powder was vacuum packed in polyethylene bags and stored at −80 °C until vitamin C extraction. Samples for thermal treatments were prepared by freezing a portion of the remaining florets in liquid nitrogen and subsequently crushing them into a frozen powder, which was vacuum packed in polyethylene bags and stored at −80 °C until thermal treatments. The samples were then thawed for 7 min at 25 °C and then heat treated for 15 min in a thermostated water bath (W14 Grant Instruments, Cambridge, England) at 30 to 90 °C. The thermal-treated samples were immediately cooled in ice water, frozen in liquid nitrogen, and stored at −80 °C until vitamin C extraction.

Vitamin C extraction. Vitamin C (L-AA and DHAA) was extracted using the method described by Galgano and others (2007) with few modifications. Frozen broccoli samples were thawed for 5 min in a water bath at 25 °C. About 10 g thawed broccoli was mixed with 50 mL extraction solution (20 mM NaH_2PO_4, pH 2.1, 1 mM EDTA) at 4 °C and homogenized using ultra Turrax mixer (T25, Ika, Germany) at 9500 rpm for 1 min. The homogenates were then centrifuged at 29000 × *g* and 4 °C for 30 min and subsequently filtered using a quantitative filter paper (diameter 125 mm, Macherey-Nagel, Germany). The supernatant was stored at −80 °C until high-pressure liquid chromatography (HPLC) analysis. Samples from each treatment were extracted in duplicate.

HPLC analysis of vitamin C. Vitamin C was analyzed using HPLC as previously described (Munyaka and others 2010). Total vitamin C in broccoli was determined as the sum of L-AA and DHAA. The DHAA content was determined after precolumn reduction to L-AA using Tris (2-carboxy-ethyl) phosphine HCl (TCEP–HCl) solution. The DHAA content was determined as the difference between L-AA content after and before reduction. L-AA in broccoli was quantified using an external calibration curve constructed using L-AA standard solution (50 μg/mL L-AA dissolved in NaH_2PO_4 [20 mM, pH 4.0, 1 mM EDTA]). The 8-point calibration curve (L-AA concentration range 0.25 to 2.5 μg on column) used for quantification was linear ($R^2 = 0.9999$). The quantification was based on peak area.

Study on the thermal stability of AAO

Broccoli florets and stalks were crushed for 10 s using a Buchi mixer (B-400, Flawil, Switzerland) and immediately placed in ice. For thermal treatments, 14 g aliquots of crushed floret and stalk material were packed in stainless steel tubes (100 mm length, 12 mm diameter). The temperature of the samples was brought to 20 °C by immersion for 4 min in a water bath (WBU-45, Memmert, Germany) at 20 °C. The thermal stability of AAO was screened by heating the samples for 10 min in a water bath at 20 to 95 °C (intervals of 5 °C). A detailed study on the thermal inactivation kinetics of AAO in broccoli florets was performed at 55, 57.5, 60, 62.5, and 65 °C for predetermined time intervals in a thermostated water bath. After thermal treatments, samples were immediately cooled in ice water. During thermal treatments, the temperature of the samples was followed using thermocouples connected to a temperature-recording device (Ellab PA-9600, Hilleroed, Denmark).

AAO assay. AAO activity in broccoli was determined using the method described by Oberbacher and Vines (1963) with some modifications. To extract AAO, 7.5 g broccoli was mixed with 15 mL phosphate buffer (0.1 M, pH 5.6, 0.5 mM EDTA) and homogenized for 30 s, followed by centrifugation of the homogenates for 30 min at 17900 × *g* and 4 °C. The resulting supernatant was filtered using 0.45 μm cellulose filter (Macherey-Nagel, Duren, Germany) and stored in ice prior to enzyme activity measurements. During extraction, the effect of including different concentrations of NaCl (0 to 1.5 M) and triton X-100 (0% to 1.5%) in the extraction buffer on AAO extraction yield was evaluated. The presence of 0.75 M NaCl in the extraction buffer resulted in 5-fold increase in AAO extraction yield and therefore this concentration of NaCl was always included in the extraction buffer.

AAO activity was determined by measuring for 3 min, the decrease in substrate concentration (5 mM L-AA) using a spectrophotometer (Ultrospec 2100 Pro, Biochrom, Cambridge, UK) at 25 °C and 265 nm. The assay mixture consisted of 2.85 mL phosphate buffer (0.1 M, pH 5.6, 0.5 mM EDTA), 75 μL substrate, and 75 μL enzyme extract in a 10 mm quartz cuvette. The blank consisted of 2.925 mL phosphate buffer (0.1 M, pH 5.6, 0.5 mM EDTA) and 75 μL AAO extraction buffer (0.1 M, pH 5.6, 0.5 mM EDTA, 0.75 M NaCl). One unit of enzyme was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ascorbic acid per minute at 25 °C.

Data analysis. In this study, AAO thermal inactivation data were modeled using a two-step nonlinear regression analysis using SAS 9.1.3 service pack 3 (SAS 2002–2003, SAS Institute Inc., Cary, N.C., U.S.A.). The thermal inactivation rate constant (k) for AAO in broccoli was estimated using a 1st order kinetic model whereas the temperature dependence (activation energy, E_a) of k values was estimated using the Arrhenius equation.

Study on thermal stability of L-AA after enzyme inactivation

This study was aimed at investigating the thermal stability of L-AA in crushed broccoli in the absence of enzymatic reactions. Crushed broccoli powder was prepared from florets blanched before crushing as described in the study on the thermal stability of L-AA in raw crushed broccoli. About 50 g of the frozen broccoli powder was vacuum packed in polyethylene bags and thawed for 7 min in a water bath at 25 °C. The thawed material was then heated for 15 min at 30 to 90 °C. After the thermal treatments, samples were immediately cooled in ice water, frozen in liquid nitrogen, and stored at −80 °C until vitamin C (L-AA and DHAA) extraction in the same way as described in the study on the thermal stability of L-AA in raw crushed broccoli.

Results and Discussion

Thermal stability of L-AA in raw crushed broccoli

The influence of the different thermal treatments on L-AA and DHAA concentration in broccoli florets is shown in Figure 1A. Broccoli blanched before crushing was used as reference to compare the effect of different thermal treatments on vitamin C concentration because this sample showed the highest vitamin C content and excluded interference from enzymatic conversions during analysis, as was previously demonstrated (Munyaka and others 2010). The blanching treatment was assumed to completely inactivate AAO and other enzymes because it was previously demonstrated that temperatures in the range of 92 to 96 °C could result in more than 99% inactivation of peroxidase (the most heat resistant food quality degrading enzyme) in broccoli (Murcia and others 2000; Morales-Blancas and others 2002).

A total vitamin C concentration amounting to 113.8 mg/100 g fresh weight (FW) was determined in the blanched broccoli. This value is within the range of 77.8 to 129.5 mg/100 g FW reported in literature for fresh broccoli florets (Howard and others 1999; Galgano and others 2007; Munyaka and others 2010). The percent DHA content (6.2%) found in the blanched broccoli was also in the range of 5.4% to 10.9% reported in literature for fresh broccoli florets (Wills and others 1984; Vanderslice and others 1990; Munyaka and others 2010). In this study, it was observed that vitamin C in the untreated and 30 to 60 °C-treated samples of crushed broccoli occurred entirely as DHAA. However, the blanched sample and crushed broccoli treated at 70, 80, and 90 °C contained mainly L-AA. The high percentage of DHAA in the untreated and 30 to 60 °C treated samples was attributed to enzyme-catalyzed oxidation of L-AA to DHAA whereas the retention of L-AA in 70 to 90 °C treated samples was attributed to faster and probably complete inactivation of oxidative enzymes.

Relative to the blanched sample, the untreated and treated (30 to 90 °C) crushed broccoli samples contained 21% to 43% less vitamin C (L-AA + DHAA). DHAA is reported to be more unstable (especially at low acid pH conditions typical of vegetables such as broccoli) than L-AA and easily and rapidly undergoes hydrolytic cleavage to form 2,3-DKG, a compound that does not possess antiscorbutic activity. Further degradation of 2,3-DKG either spontaneously or in the presence of oxidative species results in formation of numerous compounds including oxalate and tartarate (Hancock and Viola 2005). In this study, degradation of DHAA to other compounds could have contributed to the decrease in total vitamin C observed in the untreated and treated crushed broccoli.

Thermal stability of AAO in broccoli

The effect of thermal treatments on AAO stability in crushed broccoli florets and stalks is shown in Figure 2. In this study, AAO activity was found to be 7.5 times higher in florets than in stalks, an observation that could explain the previous (Munyaka and others 2010) observation of better L-AA retention in broccoli stalks than in florets during various treatments. Other authors (Nishikawa and others 2001, 2003; Galgano and others 2007) also reported that the vitamin C content decreased in broccoli florets but not in stalks during postharvest storage of broccoli at 20 and 6 °C, respectively. In this study, AAO in crushed broccoli florets and stalks was stable until around 50 °C but was almost completely inactivated after a 10 min thermal treatment at 80 °C. It was concluded that AAO inactivation was irreversible since no increase in activity was observed when extracts of thermal treated broccoli were stored at 4 °C for 24 h (data not shown).

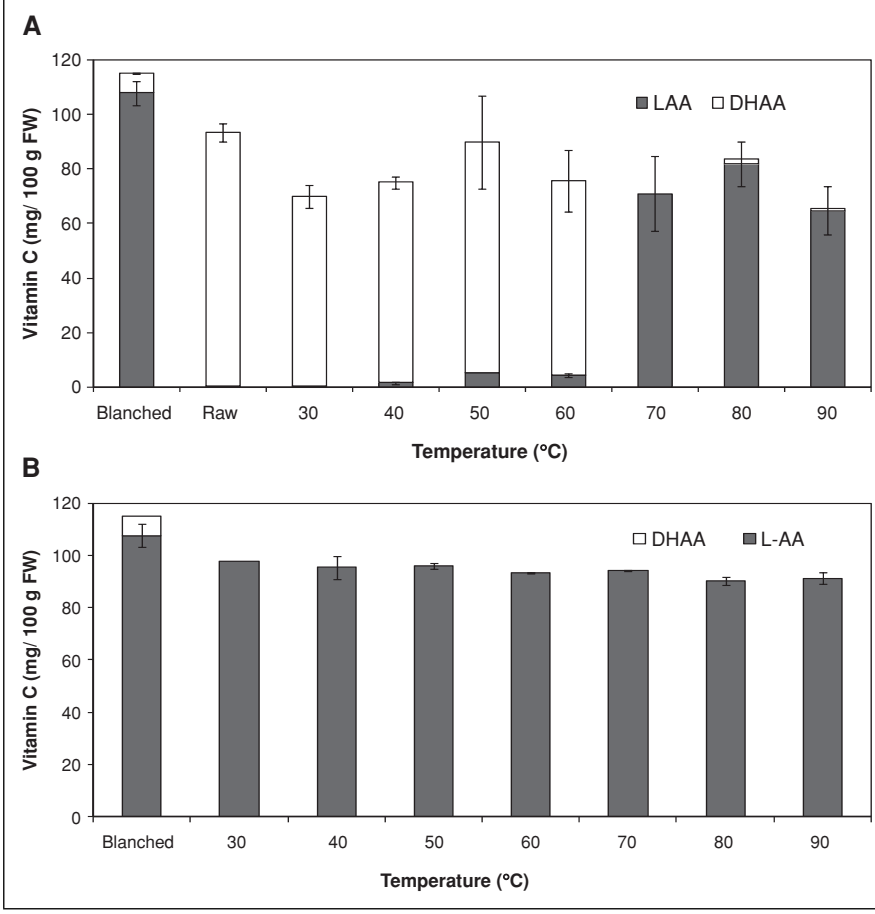

Figure 1 — Effect of thermal treatments (15 min) on vitamin C concentration in (A) broccoli florets crushed in the raw state and (B) broccoli florets crushed after blanching. Values are mean ± standard deviation of duplicate analysis.

Because broccoli florets showed higher AAO activity compared to stalks, the former tissue was used for further studies on thermal inactivation kinetics. The effect of different temperature/time combinations on AAO stability in crushed florets is shown in Figure 3. In this study, it was observed that AAO activity in broccoli exhibited a temperature/time-dependent increase during the nonisothermal region (approximately 4 min) of heating at 57.5 to 65 °C, before the onset of inactivation. The observed increase in AAO activity was attributed to increased enzyme extractability due to cell wall breakdown at high temperatures, in accordance with a previous report (Kim and others 1996; Liso and others 2004) that AAO is mainly bound to the cell wall. The localization of AAO in the cell wall was further supported by observation of increased enzyme extraction yield after inclusion of 0.75 M NaCl in the extraction buffer in this study. As shown in Figure 3, the rate of AAO inactivation increased with temperature and the inactivation data fitted 1st order kinetics. Estimating the inactivation rate constant (k) using a 1st order kinetic model resulted in k values (±standard error of regression) of 1.60 ± 0.12, 3.35 ± 0.13, 9.04 ± 0.46, 23.53 ± 0.93, and (40.81 ± 2.75) × 10^{-2} min^{-1} for 55, 57.5, 60, 62.5, and 65 °C, respectively. The temperature dependence (activation energy, E_a) of k was estimated using the Arrhenius equation (Figure 4) and a value of 266.57 ± 6.03 kJ/mol was determined.

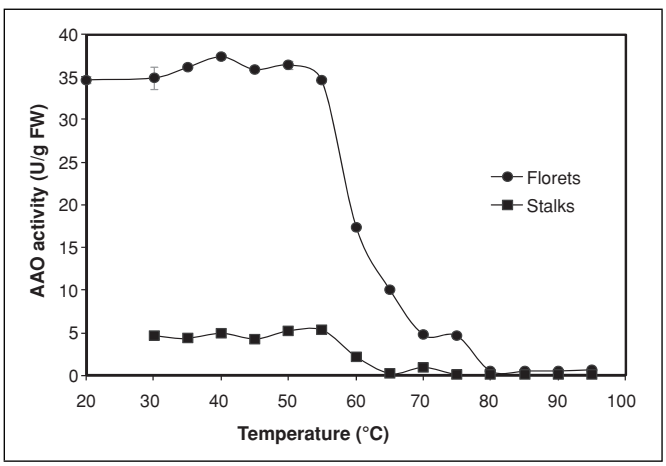

Figure 2—Influence of thermal treatments (10 min) on AAO activity in crushed broccoli florets and stalks. Values are mean ± standard deviation of duplicate measurements.

Thermal stability of L-AA in broccoli crushed after enzyme inactivation

The influence of thermal treatments on broccoli crushed after blanching is shown in Figure 1B. In this study, it was observed that vitamin C in samples treated at 30 to 90 °C occurred as L-AA. Compared to the untreated blanched sample, blanched samples treated at 30 to 90 °C showed overall vitamin C losses amounting to 19% to 25%, which were partly attributed to the degradation of the initially present DHAA. These losses were lower than those (21% to 43%) resulting from thermal treatment of raw crushed broccoli. Considering that samples blanched prior to thermal treatment had received more total heating (and yet retained more vitamin C) than samples thermally treated before blanching, it was concluded that enzyme-catalyzed oxidation of L-AA to DHAA could be the starting point of L-AA degradation in vegetables such as broccoli. The results of this study were in agreement with the results of a previous study (Munyaka and others 2010) that samples blanched (high temperature short time [90 °C/4 min] and low temperature long time

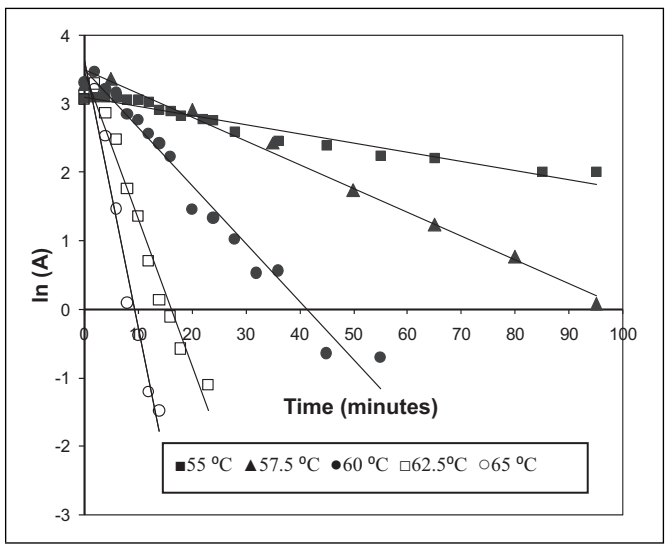

Figure 3—Effect of thermal treatments on AAO activity (logarithmic scale) in crushed broccoli florets. Full lines (fitted curves according to 1st order kinetics model) showed the following R^2 values: 55 °C (R^2 = 0.94), 57.5 °C (R^2 = 0.99), 60 °C (R^2 = 0.97), 62.5 °C (R^2 = 0.97), and 65 °C (R^2 = 0.98).

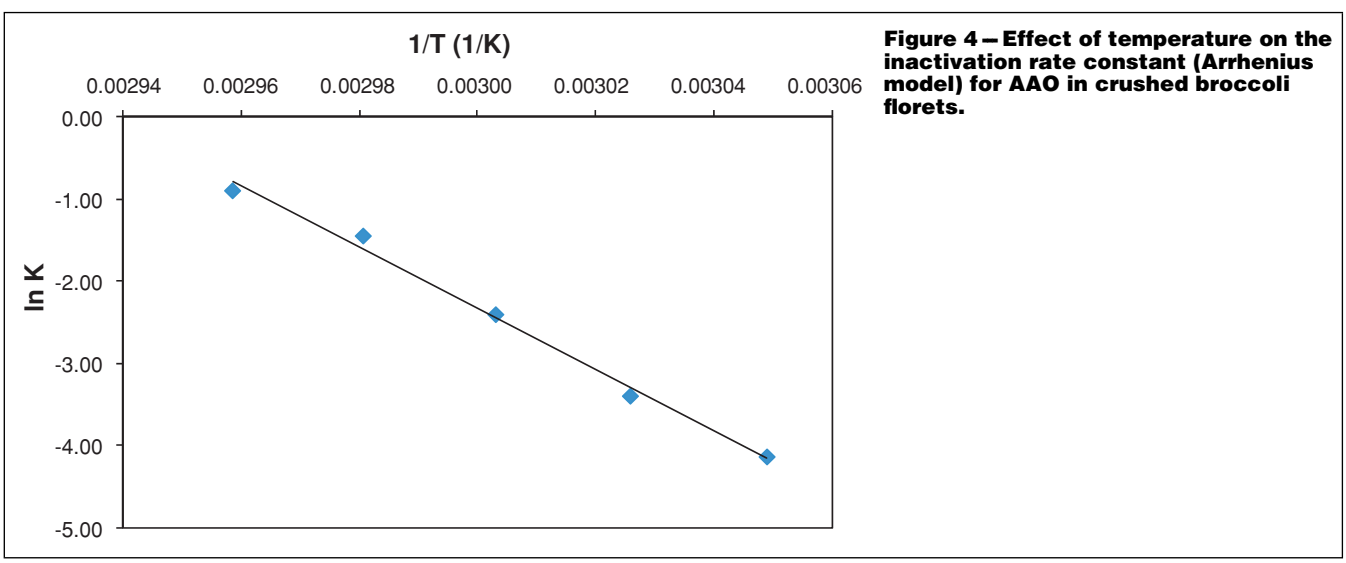

Figure 4—Effect of temperature on the inactivation rate constant (Arrhenius model) for AAO in crushed broccoli florets.

[60 °C/40 min]) before crushing retained more vitamin C and contained less DHAA than those crushed before blanching.

Conclusions

Literature information on the effect of different temperatures on the concentration of L-AA and DHAA, and the stability of AAO during vegetable processing is scarce. This study showed that L-AA could entirely be enzymatically oxidized to DHAA during processing of crushed vegetable products (for example, juices and purees) at temperatures below 70 °C, with the implication that serious loss of vitamin C activity upon further degradation of DHAA could occur. In this study, it was observed that AAO activity in broccoli florets was 7 times higher than in broccoli stalks, indicating that enzymatic conversions of L-AA to DHAA could be higher in florets than in stalks. Although thermal inactivation was studied only for AAO in this study, the inactivation of other L-AA oxidizing enzymes such as APx should also be investigated because their role in L-AA degradation could not be excluded in this study.

Acknowledgments

The support of the following organizations in accomplishment of this work is acknowledged: Interfaculty Council for Development Co-operation (IRO-Doctoral scholarship, K.U. Leuven), Specific Targeted Research Project (EU Project, Healthy Structuring 2006–023115), and Research Foundation Flanders (Postdoctoral grant, FWO-Vlanderen).

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