J. Appl. Glycosci., **71**, 1−7 (2024)
doi:10.5458/jag.jag.JAG-2023_0009

Regular Papers

Promotion of Thermal Inactivation Treatment of Apple Polyphenol Oxidase in the Presence of Trehalose

(Received September 4, 2023; Accepted November 7, 2023)

Shinya Yamazaki,[1,2,†] Ibuki Shirata,[2] Masahiro Mizuno,[2] and Yoshihiko Amano[2]

[1] *Food Technology Department, Nagano Prefecture General Industrial Technology Center* (*205−1 Kurita, Nagano, Nagano 380−0921, Japan*)
[2] *Graduate School of Medicine, Science and Technology, Department of Biomedical Engineering, Shinshu University* (*4−17−1 Wakasato, Nagano, Nagano 380−8553, Japan*)

Abstract: Trehalose is known to protect enzymes from denaturation. In the present study, we observed promotion of apple polyphenol oxidase (PPO) inactivation in a trehalose solution with thermal treatment. Crude PPO from Fuji apple was mixed with either sucrose or trehalose solutions, then the samples treated at 25 or 65 ˚C. In the presence of trehalose, PPO activities were markedly decreased upon treatment at 65 ˚C with increasing trehalose concentration. Furthermore, the reduction in PPO activity in the presence of trehalose was proportional to storage time after thermal treatment and thermal treatment time. Comparing PPO activities between treatment time 0 and 90 min at 65 ˚C, activities decreased 89 % for trehalose concentration of 0.2 M. These results indicates that trehalose acts not only as inhibitor but as promoter of inactivation of PPO. The Lineweaver–Burk plot indicated that trehalose acts on PPO as a non-competitive inhibitor during the 65 ˚C treatment. Two mechanisms of PPO inactivation in the presence of trehalose were suggested; one is the suppression of PPO activation cause by a thermal treatment, and another is the conformational change to inactivation form of PPO in conjunction with trehalose and a thermal treatment. Additionally, apple juice including 0.2 or 0.5 M trehalose with 65 ˚C treatment indicated slow browning than the juice with 0.2 or 0.5 M sucrose or without sugars. This result demonstrates that the preventing of browning with trehalose is a viable industrial food process.

Key words: trehalose, polyphenol oxidase, apple, thermal treatment, inactivation

INTRODUCTION

Polyphenol oxidase (PPO) (E.C. 1.10.3.2) occurs naturally in fruits, vegetables, fungi, and animals [1].This enzyme oxidizes mono- or di-phenol compounds to *o*-quinones, forming brown polymers resulting in undesirable color changes and food degradation [2]. Although thermal treatment is the primary method for PPO inactivation, it requires either high temperatures or long treatment times [3]. High temperature treatments often negatively impact food quality, causing undesirable features such as texture softening, flavor loss, and destruction of thermosensitive nutrients such as vitamins [4]. Therefore, PPO inhibitors are alternatives to prevent browning using non-thermal treatments, for example, reducing agents, acids, chelating additives, halides, peptides, and higher alcohols [2]. Ascorbic acid [5], L-cysteine [6], halides, such as sodium chloride [7], phytic acid [8], glutathione [9] and kojic acid [10] are well known PPO inhibitors. However, these treatments may also induce undesirable changes to food taste, and some are forbidden to be used as food additives. Though high hydrostatic pressure treatment is known as non-thermal treatment which is effective on food pasteurization, high hydrostatic pressure treatment is not enough to completely inactivate PPO activity [11].

Sugars are widely used in food processing to influence taste, color, and texture. Some sugars are also reported to have an inhibiting effect on PPO such as β-cyclodextrin [12], L-arabinose [13] and 1,5-anhydro-D-fructose [14]. However, these sugars are difficult to use in food processes as food inhibitors due to their potentially high-cost or limited concentrations allowed for food additives. Sucrose and trehalose are frequently used in food processes for seasoning, improved shelf-life, and appearance. Trehalose is particularly well known for various effects such as protein stabilization, inhibition of starch retrogradation, and as a cryoprotectant [15,16]. Trehalose is also known to prevents enzyme denaturation [17,18]. However, in the present study, we investigated the effect of sugars in conjunction with thermal treatment on PPO activity and found a novel inhibition of trehalose for PPO activity.

MATERIALS AND METHODS

Chemicals and Reagents. The reagents sucrose, trehalose dihydrate, pyrocatechol, ascorbic acid (reagent grade, respectively), and ammonium sulfate (enzyme refining) were purchased from FUJIFILM Wako Pure Chemical

†Corresponding author (Tel. +81−26−227−3131, Fax. +81−26−227−3130, E-mail: yamazaki-shinya-r@pref.nagano.lg.jp)
Abbreviations: cPPO, crude PPO solution; PMSF, phenylmethylsulfonyl fluoride; PPO, polyphenol oxidase; PVPP, polyvinylpolypyrrolidone.

Corporation (Osaka, Japan). Polyvinylpolypyrrolidone (PVPP) (extra pure grade) was purchased from NACALAI TESQUE, Inc. (Kyoto, Japan). Phenylmethylsulfonyl fluoride (PMSF) (for biochemical research) was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan).

Raw Materials. Fuji apples (*Malus domestica. cv. Fuji*) were purchased from a fruit and vegetable store in Nagano city, Nagano prefecture, Japan, and stored at 2−4 ˚C. Apples were cored, sliced and frozen at –40 ˚C by rapid freezer (QXF-006SFSV1, Fukushima Galile, Japan). Then the frozen apples were lyophilized and powdered.

Crude PPO purification. Soluble PPO was purified according to the method of Han *et al.* [19]. All purification operations were performed at 4 ˚C. A 40 g sample of lyophilized apple powder was mixed with 600 mL of ice cold 50 mm sodium phosphate buffer (pH 6.8), containing 2 % (w/v) PVPP and 1 mM PMSF. The mixture was centrifuged at 10,800 × G for 30 min. The supernatant was separated and dissolved ammonium sulfate to 50 % saturation. After stirring for 24 h, the solution was centrifuged at 10,800 × G for 20 min. The supernatant was separated and brought to 80 % saturation of ammonium sulfate, stirred for 24 h, and centrifuged again at 10,800 × G for 20 min. The precipitate was separated and dissolved in a minimum volume of 50 mM sodium phosphate buffer (pH 6.8), and dialyzed through a 12−14 kDa cut-off membrane in 3 L of 50 mM sodium phosphate buffer (pH 6.8) with three buffer changes over the 24-h period. The dialyzed solution was used as the crude PPO solution (cPPO) for subsequent experiments.

Thermal treatment of PPO with sugars. Sugar solutions were prepared with 0.1−0.4 M trehalose or sucrose dissolved in 50 mM sodium phosphate buffer (pH 6.8). Sugar solutions and cPPO were mixed in a 1:1 ratio (v/v), then the mixtures dispensed to 1.5 mL tubes. The tubes were treated at 25 or 65 ˚C for 30−180 min in a water bath, with subsequent immediate ice bath cooling. The samples were then immediately measured for activity (0 day). Furthermore, samples treated for 30 min were stored at 4 ˚C for 1−3 day with activity measured daily.

Determination of inhibition type. The type of inhibition was determined as follows. Mixed samples with 0.1−0.4 M trehalose or sucrose solutions were treated at 25 or 65 ˚C for 30 min, then cooled in ice bath as previously. The treated samples were immediately evaluated for enzyme activity using 5−50 mM of pyrocatechol dissolved in 50 mM sodium phosphate buffer (pH 6.8) as the substrate. Plotting the inverse of enzyme activity ($1/V$) against the inverse of substrate concentrations ($1/S$), the inhibition type was estimated by the Lineweaver–Burk plot.

The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined by linear regression of the Lineweaver–Burk plots, with the inhibition constant (K_i) determined from linear regression of the Dixon plot. The Dixon plot consisted of the inverse of enzyme activity ($1/V$) against the inhibitor concentration (I) from the treatment at 65 ˚C with trehalose.

Enzyme assays. Enzyme assays were performed according to Han *et al*. [19] with slight modification. The substrate solution, 20 mM pyrocatechol, was prepared by dissolving the substrate in 50 mM sodium phosphate buffer (pH 6.8). A 20 μL aliquot was extracted from the thermal treatment tubes and added to a 96-well microplate and incubated at 25 ˚C for 1 min. Then, 180 μL substrate solution was added to the 96-well microplate. Enzyme activity was then measured by changes in the 410 nm absorption at 25 ˚C every 5 s for a total duration of 90 s with a multi detection microplate reader (Varioskan Flash, Thermo Fisher Scientific, USA) in triplicate. Enzyme activity was defined as a 0.01 absorbance change in the initial linear part (ΔA/min) per 1 mL of enzyme solution.

Preventing apple juice browning. Apple juice was prepared as follows: Fuji apples were peeled and cored, then cut into eight pieces. Each piece was then cut into 1-cm thick slices. The sliced apples were mixed with a 1.5 % ascorbic acid solution in the ratio of 100 g apple to 2 mL ascorbic acid solution, followed by squeezing using a hand press juicer.

A 0.2 M and 0.5 M solution of trehalose or sucrose for dissolving in the juice was prepared, then juices with sugars and without sugars were put into 25 mL tubes and heated in a water bath at 65 ˚C over 30 min followed by immediate cooling in water. Then, 20 mL of each treated juice was placed in a beaker and stirred using magnetic stirrer at 200 rpm under ambient conditions (24−26 ˚C) with color measurements taken every 0.5 h to 2 h. Water activity of the juices with the 0.2 M and 0.5 M of trehalose or sucrose were measured by water activity meter (LabMaster-aw, novasina AG, Switzerland).

Color measurements. Color measurements using the CIE $L^*a^*b^*$ system were obtained using a spectrophotometer (SE 7700, Nippon Denshoku Industries, Tokyo, Japan). Instrument calibration used a white color standard, with juice samples placed in a glass cell, measured in transmission mode, in triplicate. The degree of browning was expressed as the total color differences (ΔE^*), calculated as follows:

$$\Delta E^* = \sqrt{\left(L^* - L_0^*\right)^2 + \left(a^* - a_0^*\right)^2 + \left(b^* - b_0^*\right)^2} \quad (1)$$

Where L^*, a^*, and b^* correspond to the color values of time elapsed samples (0.5−2 h), L_0^*, a_0^*, and b_0^* correspond to samples before time elapsed (0 h).

Statistical analysis. Results are shown as the mean ± standard deviation ($n = 3$). Statistical analysis was conducted using the Tukey test. The test was performed using Microsoft Excel 2016 (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Effect of thermal treatment with trehalose on PPO inactivation.

PPO was mixed with trehalose or sucrose, and treated at 25 or 65 ˚C for 30 min. The sugar concentrations were 0, 0.05, 0.1, 0.2 M. Figure 1 shows PPO activities after thermal treatment at 25 ˚C (Fig. 1A) or 65 ˚C (Fig. 1B). PPO activities at 25 ˚C were almost unchanged for all sugar concentrations compared to the absence of sugar. For the thermal treatment at 65 ˚C, PPO activity, in the absence of sugars, was about 3-fold higher than for the 25 ˚C treatment. It is reported that thermal treatment of about 70 ˚C activates PPO [20,21]. The increased PPO activity observed here agrees with these reports. In the presence of sucrose, PPO activity was unchanged or slightly decreased compared to no sugar. On the other hand, in the presence of trehalose, PPO activity

was significantly decreased with increasing trehalose concentrations. For 0.2 M trehalose, the activity was 29 % lower than that in the absence of sugars.

The thermally treated mixtures were stored at 4 ˚C for 3 days. Figure 2 shows the changes in PPO activity of the samples after storage. In the absence of sugars, PPO activity treated at 25 ˚C decreased by approximately 19 %. In the presence of sugars, the activities decreased similarly for each sugar. The PPO activities were decreased by 25−36 %. For the thermally treated (65 ˚C) samples, PPO activity in the absence of sugars was slightly decreased, with a 13 % reduction. In the presence of sucrose, PPO activity decreases were almost the same as with the 25 ˚C treatment, with reductions of 24 and 33 % in 0.1 and 0.2 M, respectively. In the presence of trehalose, PPO activity showed higher reductions. In the presence of 0.1 M trehalose, the PPO activity with the 25 ˚C treatment exhibited a 32 % reduction. In the presence of 0.2 M trehalose, PPO activity was decreased by 89 %. This demonstrated that high trehalose concentrations with 65 ˚C treatment strongly inhibit PPO activity during storage.

Figure 3 shows PPO activity after thermal treatments of 25 ˚C (Fig. 3A) and 65 ˚C (Fig. 3B), with sugar solutions of different concentrations over time. PPO treated at 25 ˚C for 30−180 min in the absence of sugars resulted in a final PPO activity reduction by 20 %. In the presence of trehalose or sucrose, PPO activities decreased 7−16 % after 180 min. These results indicate that the sugars suppressed PPO inactivation at 25 ˚C. On the other hand, at 65 ˚C, PPO activities increased 2.4−2.5-fold from 0 to 30 min in treatments without trehalose. However, in the presence of 0.1 M trehalose, the PPO activity only increased 1.6-fold. Furthermore, in 0.2 M trehalose, PPO activity decreased by about 15 %. From 30 to 90 min, PPO activity in the absence of sugars or presence of sucrose gradually decreased with treatment time. These reductions were 29–35 % from 30 to 90 min. On the other hand, in the presence of trehalose, PPO activities decreased 77 and 87 % from 30 to 90 min at the concentrations of 0.1 and 0.2 M, respectively. Comparing PPO activities between treatment time 0 and 90 min, activities decreased 62 and 89 % for trehalose concentrations of 0.1 and 0.2 M, respectively. In contrast, after 90 min, PPO

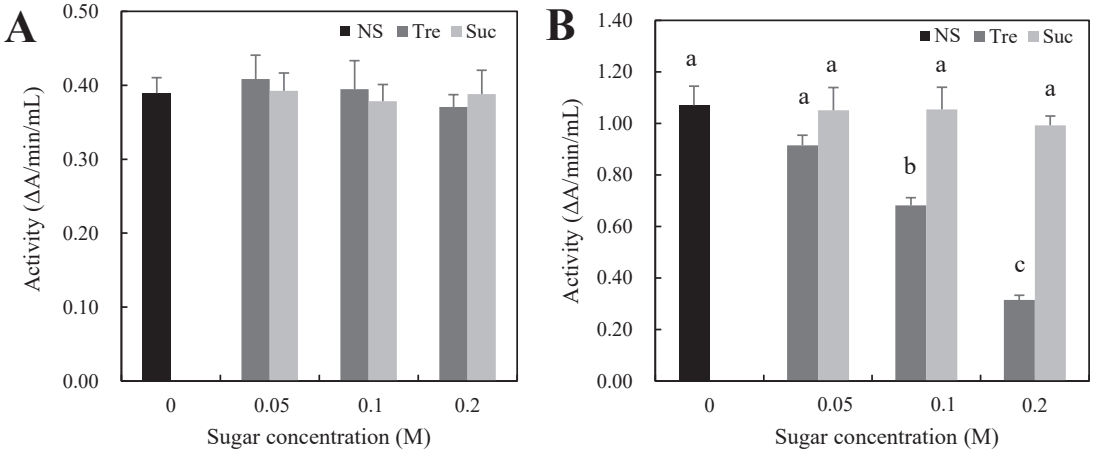

Fig. 1. PPO activities with trehalose or sucrose (0.05−0.2 M), or without sugars (0 M).
(A) shows the treatment at 25 ˚C for 30 min and (B) shows the treatment at 65 ˚C for 30 min. Values with different letters (a−c) indicate significant differences of each group of same treatment temperatures ($p < 0.05$). Values with same or no letters indicate no significant differences ($p < 0.05$). Error bars represent standard deviation (n=3). (NS: without sugars, Tre: trehalose, Suc: sucrose).

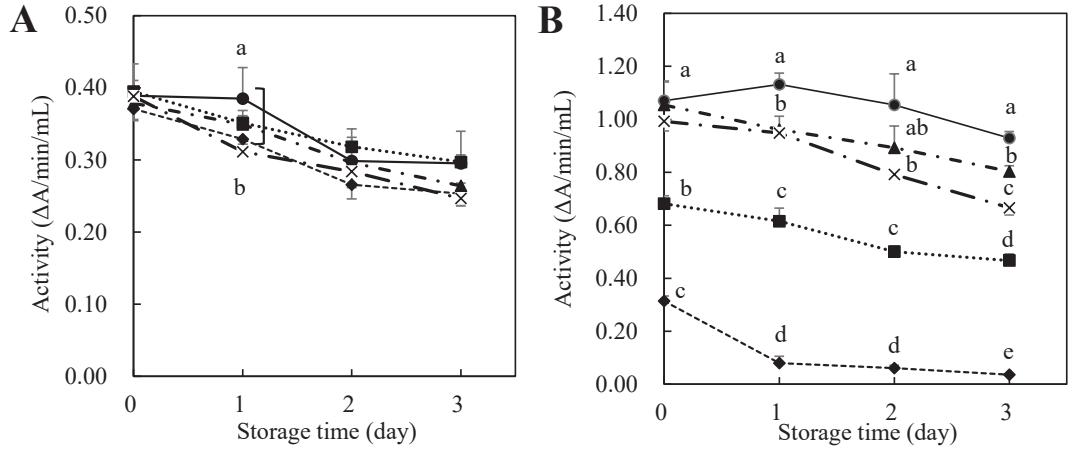

Fig. 2. Activities of PPO treated with 0.1 (■) or 0.2 M (◆) trehalose and 0.1 (▲) or 0.2 M (×) sucrose, or without sugars (●).
These were stored for 3 days at 4 ˚C. (A) shows the treatment at 25 ˚C for 30 min and (B) shows the treatment at 65 ˚C for 30 min. Activities of storage time day 0 in (A) and (B) are same data as Figs. 1A and 1B. Values with different letters (a−e) indicate significant differences of each group of same storage times ($p < 0.05$). Values with same or no letters indicate no significant differences ($p < 0.05$). Error bars represent standard deviation (n=3).

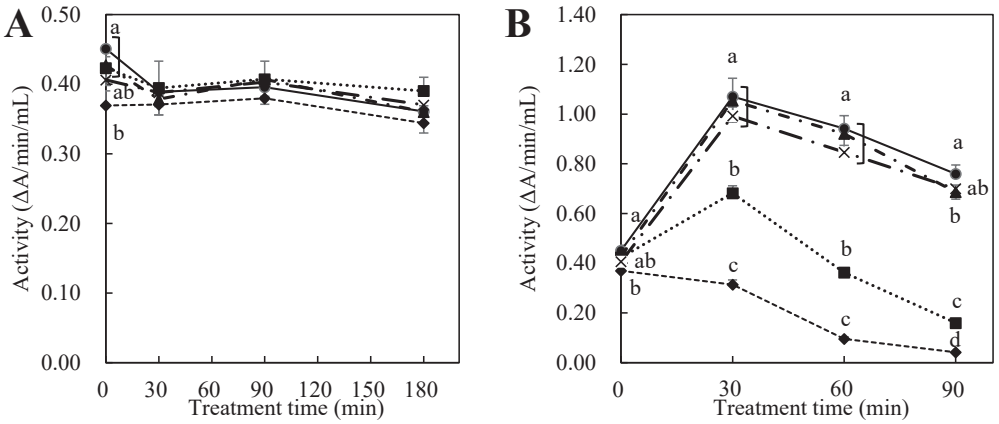

Fig. 3. Activities of PPO treated with 0.1 or 0.2 M trehalose and 0.1 or 0.2 M sucrose, or without sugars. The symbols are same as Fig 2.

(A) shows the treatment at 25 ˚C and (B) shows the treatment at 65 ˚C for 30−180 min. Activities of treatment time 0 min are same data as (A) and (B). Values with different letters (a−d) indicate significant differences of each group of same treatment time ($p < 0.05$). Values with same or no letters indicate no significant differences ($p < 0.05$). Error bars represent standard deviation (n=3).

activities in the absence of sugars or presence of sucrose were 1.6−1.7-fold higher than at 0 min.

Trehalose is a known thermoprotectant for enzymes [15,16] with Gheibi *et al*. [22] reporting the trehalose stabilization of mushroom tyrosinase at both 4 and 40 ˚C. However, in the present study, trehalose promoted PPO inactivation with thermal treatment, behavior contrary to that normally reported. Only a few studies reported enzyme inactivation in the presence of trehalose. Habib *et al*. [23] found that sucrose and trehalose promoted bromelain inactivation with thermal treatment at 60 ˚C, and trehalose was more effective than sucrose. Habib suggested that sucrose/trehalose are preferentially hydrated over the denatured bromelain compared with the native enzyme, the unfolded conformation becomes stabilized in the presence of sucrose/trehalose.

We suggest a PPO inactivation mechanism upon thermal treatment in the presence of trehalose as follows. PPOs are known to become activated under certain thermal treatment conditions [20,21]. Murtaza reported apple PPO secondary structure conformational changes upon treatment at 70 ˚C for 10 min, and the subsequent activity was higher than the native conformation. On the other hand, Miroliaei *et al*. [24] found that trehalose-induced thermal protection of the α-helix section of yeast alcohol dehydrogenase. Figure 3 shows that, in the absence of sugars or the presence of sucrose, thermal treatment at 65 ˚C for 30 min increased PPO activity. However, in the presence of trehalose, this thermal activation of PPO at 65 ˚C was suppressed. It is presumed that the thermally induced PPO conformational changes to a conformer of increased activity was suppressed in the presence of trehalose. Additionally, Figs. 2 and 3 show further PPO activity reductions with storage time after the 65 ˚C treatment or longer treatment times at 65 ˚C. These results indicate that trehalose not only suppresses PPO activity but promotes PPO inactivation with thermal treatments.

Inhibition type of trehalose with thermal treatment.

To determine the inhibition type of sugars with thermal treatment of PPO, the Lineweaver–Burk plot was performed (Fig. 4). PPOs with thermal treatment at 25 ˚C of both sugars (Figs. 4A and 4B), and 65 ˚C in the presence of sucrose (Fig. 4C) yielded similar K_m and V_{max} values at any concentration, which indicates non- or slight inhibition. On the other hand, PPO treated at 65 ˚C with trehalose (Fig. 4D) showed an increase in gradient with increasing trehalose concentrations. This inhibition type was regarded as non-competitive as it intersects at a point on the X axis. For the 65 ˚C treatment with trehalose, V_{max} was 2.9, 2.0, 1.8 and 0.81 (ΔA/mL/min) for trehalose concentrations of 0, 0.05, 0.1 and 0.2 M, respectively. K_m was the same as for the non-competitive type inhibitors, and 23.8 ± 3.1 mM. K_i was calculated from the Dixon plot (Fig. S1; see J. Appl. Glycosci. Web site), yielding a value of 71 ± 13 mM.

The inhibitory effects of sugars against PPO or tyrosinase are described in literature. Hu *et al*. [13] reported that L-arabinose inhibits tyrosinase when using 3-hydroxy-L-tyrosine (L-DOPA) as a substrate due to binding with amino acid residues inside the tyrosinase active site. Parrilla *et al*. [12] as also reported the β-cyclodextrin inhibition of apple PPO. However, these sugars are suggested to function as mixed type inhibitors of the enzymes. Figure 4 shows trehalose functioning as a non-competitive type inhibitor to PPO at 65 ˚C. Hence, trehalose has a different mode of inhibition than these sugars for PPO inactivation. Maillard reaction products are known to inhibit PPOs and function as non-competitive type inhibitors [25]. However, trehalose is non-reducibility sugar, which does not generate maillard products. The second mechanism of PPO inactivation is assumed to relate to the structural changes of PPO upon thermal treatments to more inactive states in the presence of trehalose. Sampedro and Uribe [26] described that high viscosity with trehalose protects proteins from unfolding during heat shock, whereas viscosity increasing under cold conditions inhibits enzyme activity. Trehalose inhibited *Kluyveromyces lactis* H^+-ATPase activity with increasing concentrations. However, Sampedro also found that glycerol and sucrose inhibit dehydrogenase by increasing viscosity. On the other hand, López-Díez and Bone [27] reported that trehalose interacts more strongly with proteins than sucrose, additionally, at high temperatures trehalose interactions are stronger and sucrose interactions are weaker. Although this

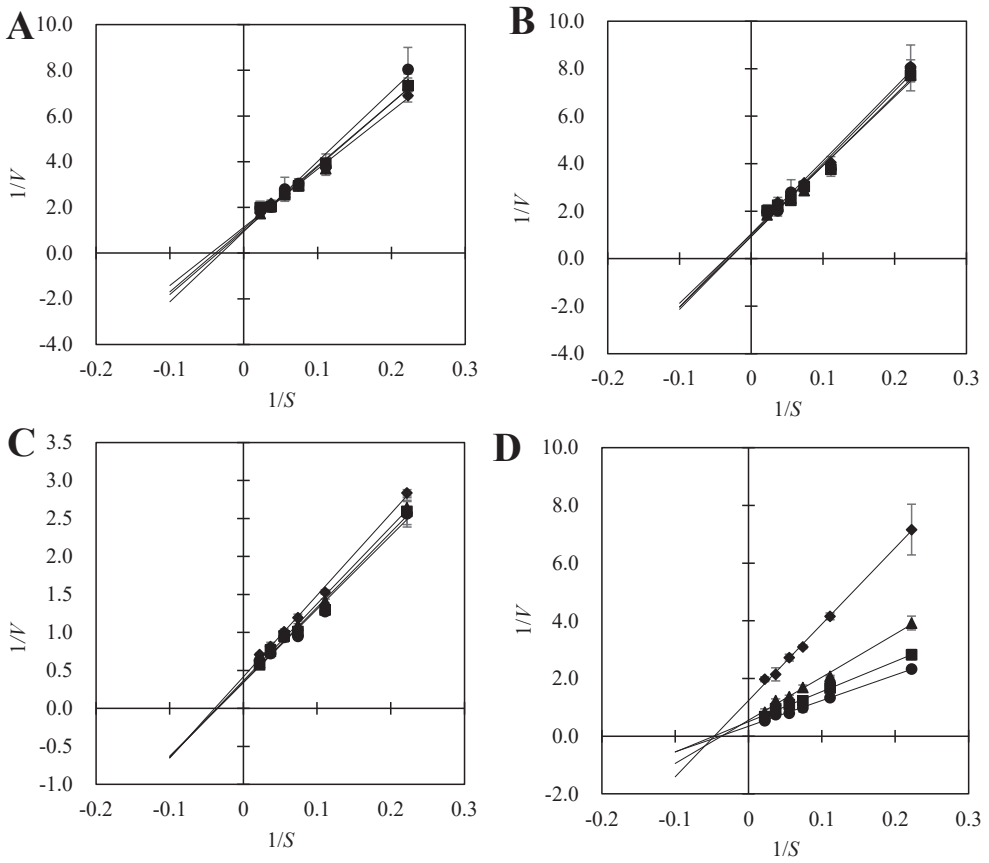

Fig. 4. Lineweaver-Burk plots for PPO activity inhibition by treatment with different sugars and temperatures for 30 min.

(A) shows the treatment at 25 ˚C with sucrose. (B) shows the treatment at 65 ˚C with sucrose. (C) shows the treatment at 25 ˚C with trehalose. (D) shows the treatment at 65 ˚C with trehalose. The concentrations of sugars are 0 (●), 0.05 (■), 0.1 (▲), and 0.2 M (◆). Error bars represent standard deviation (n=3).

phenomenon is in a solid state, these reports suggest that trehalose interacts strongly with PPO at high temperatures (65 ˚C), with the structural changes. Additionally, when PPO changes to convert to an inactivated unfolded conformation by thermal treatments, with this conformation stabilized in the presence of trehalose [23]. Figure 4 shows that trehalose inhibited PPO at 65 ˚C in a non-competitive inhibition mode. This indicates that enzymes are subjected structural changes and reaction with substrates are suppressed by the inhibitors.

Preventing browning of apple juices.

Figure 5 shows the total color differences (ΔE*) for apple juices left at room temperature for 0−2 h after the treatment at 65 ˚C for 30 min with 0.2 and 0.5 M trehalose or sucrose, or without sugars. The ΔE* of the absence of sugars was rapidly increased and reached about 3 at 2 h. The ΔE* of juices with 0.2 and 0.5 M sucrose increased with almost same tendency, and the values were reached about 2.5 at 2 h. On the other hand, ΔE* of the presence of 0.2 and 0.5 M trehalose slowly increased, then the juice with 0.5 M trehalose reached only half the value of the absence sugars at 2 h. The ΔE* of the juice with 0.2 M trehalose was higher value than the 0.5 M trehalose, whereas the value was lower than the absence of sugars or presence of sucrose at 1−2 h. These results are consistent with Fig. 1 which PPO activities with the treatment at 65 ˚C reduced with increasing concentrations of trehalose and were almost same at any concentrations of sucrose. The rate of browning is known to be

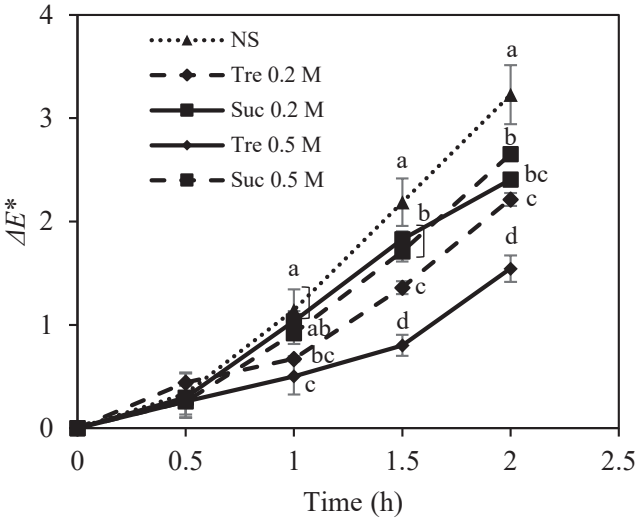

Fig. 5. The total color differences (ΔE*) of the juices incubated for 0−2 h after the treatment with or without sugars.

Values with different letters (a−d) indicate significant differences of each juice sample of same time ($p < 0.05$). Values with same or no letters indicate no significant differences ($p < 0.05$). Error bars represent standard deviation (n = 3). NS: without sugars, Tre: trehalose, Suc: sucrose.

accelerated with increased water activity [28]. In addition, trehalose and sucrose have similar water activity values at the same molarity [29]. Indeed, the value of water activities of the juices including 0.2 M of trehalose or sucrose were 0.957 (±0.005) or 0.956 (±0.003), and 0.5 M of trehalose or

sucrose were 0.943 (±0.002) or 0.947 (±0.003), respectively. It indicates that prevention of apple juice browning with trehalose is not only due to water activity but also inhibition or inactivation of PPO by trehalose.

Although these results were not as marked as the results of experiments with cPPO because of the various components included in apple juices, trehalose delayed apple juice browning. Thus, the preventing of browning with a thermal treatment in the presence of trehalose is a viable industrial food process.

CONCLUSION

Trehalose is included in various processed foods because of low-costs and some excellent characteristics such as protein stabilization, inhibition of starch retrogradation, and keep moisture of foods. In addition, it is frequently used as an inhibitor of protein denaturation. In this study, however, a novel function different from those functions as previously known was found which promoting the inactivation of PPO.

The experiments showed that PPO was inactivated significantly in the presence of trehalose when heated at 65 ℃. This result is able to lower the processing temperature required for prevention of the browning of foods made from fruits. A low temperature treatment retains fruit qualities such as texture, flavor, and thermosensitive nutrients. Food additives such as salts, acidifiers and reductants which are known as inhibitors of PPO can alter the taste of fruit to the undesirable. Trehalose has little effect on taste because trehalose has the sweetness less than half of sucrose. Therefore, prevention of browning by trehalose is expected to be a useful technology for processing of high-quality foods made from fruits and vegetables.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

[1] Mayer AM. Polyphenol oxidases in plants and fungi: going places? A review. Phytochemistry. 2006; 67: 2318−31.

[2] Yoruk R, Marshall MR. Physicochemical properties and function of plant polyphenol: A review. J Food Biochem. 2003; 27: 361−422.

[3] Ni Eidhin DM, Murphy E, O'Beirne D. Polyphenol oxidase from apple (*Malus domestica* Borkh. cv Bramley's Seedling): Purification strategies and characterization. J Food Sci. 2006; 71.

[4] Lado BH, Yousef AE. Alternative food-preservation technologies: efficacy and mechanisms. Microbes Infect. 2002; 4: 433−40.

[5] Guerrero-Beltrán JA, Swanson BG, Barbosa-Cánovas GV. Inhibition of polyphenoloxidase in mango puree with 4-hexylresorcinol, cysteine and ascorbic acid. LWT - Food Sci Technol. 2005; 38: 625−30.

[6] İyidoğan NF, Bayındırlı A. Effect of L-cysteine, kojic acid and 4-hexylresorcinol combination on inhibition of enzymatic browning in *Amasya* apple juice. J Food Eng. 2004; 62: 299−304.

[7] Lim GGF, Imura Y, Yoshimura E. Substrate inhibition competes with halide inhibition in polyphenol oxidase. Protein J. 2012; 31: 609−14.

[8] Du Y, Dou S, Wu S. Efficacy of phytic acid as an inhibitor of enzymatic and non-enzymatic browning in apple juice. Food Chem. 2012; 135: 580−2.

[9] Gacche RN, Warangkar SC, Ghole VS. Glutathione and cinnamic acid: natural dietary components used in preventing the process of browning by inhibition of polyphenol oxidase in apple juice. J Enzyme Inhib Med Chem. 2004; 19: 175−9.

[10] Chen JS, Wei CI, Marshall MR. Inhibition mechanism of kojic acid on polyphenol oxidase. J Agric Food Chem. 1991; 39: 1897−901.

[11] Yamazaki S, Kaneko S, Takahashi Y, Amano Y. Effect of citric acid and calcium chloride combined with medium high hydrostatic pressure – medium high temperature on polyphenol oxidase activity and browning in Fuji apple compote. Food Sci Technol Res. 2023; 29: 269−75.

[12] Alvarezparrilla E, Delarosa L, Rodrigogarcia J, Escobedo-gonzalez R, Mercadomercado G, Moyersmontoya E, et al. Dual effect of β-cyclodextrin (β-CD) on the inhibition of apple polyphenol oxidase by 4-hexylresorcinol (HR) and methyl jasmonate (MJ). Food Chem. 2007; 101: 1346−56.

[13] Hu WJ, Yan L, Park D, Jeong HO, Chung HY, Yang JM, et al. Kinetic, structural and molecular docking studies on the inhibition of tyrosinase induced by arabinose. Int J Biol Macromol. 2012; 50: 694−700.

[14] Abe J, Yoshinaga K. Inhibition of *Agaricus* tyrosinase by 1,5-anhydro-D-fructose and its application to anti-browning of food materials. Nippon Shokuhin Kagaku Kogaku Kaishi. 2022; 69: 9−15.

[15] Furuki T. Thermodynamic, hydration and structural characteristics of α,α-trehalose. Front Biosci. 2009;14: 3523−35.

[16] Chen A, Tapia H, Goddard JM, Gibney PA. Trehalose and its applications in the food industry. Compr Rev Food Sci Food Saf. 2022; 21: 5004−37.

[17] Jain NK, Roy I. Effect of trehalose on protein structure. Protein Sci. 2009; 18: 24−36.

[18] Olsson C, Zangana R, Swenson J. Stabilization of proteins embedded in sugars and water as studied by dielectric spectroscopy. Phys Chem Chem Phys. 2020; 22: 21197−207.

[19] Han QY, Liu F, Li M, Wang KL, Ni YY. Comparison of biochemical properties of membrane-bound and soluble polyphenol oxidase from Granny Smith apple (*Malus* × *domestica* Borkh.). Food Chem. 2019; 289: 657−63.

[20] Yemenicioğlu A, Özkan M, Cemeroğlu B. Heat inactivation kinetics of apple polyphenoloxidase and activation of its latent form. J Food Sci. 1997; 62: 508−10.

[21] Murtaza A, Muhammad Z, Iqbal A, Ramzan R, Liu Y, Pan S, et al. Aggregation and conformational changes in native and thermally treated polyphenol oxidase from apple juice (*Malus domestica*). Front Chem. 2018; 6.

[22] Gheibi N, Saboury AA, Haghbeen K, Moosavi-Movahedi AA. The effect of some osmolytes on the activity and stability of mushroom tyrosinase. J Biosci. 2006; 31: 355−62.

[23] Habib S, Khan MA, Younus H. Thermal Destabilization of Stem Bromelain by Trehalose. Protein J. 2007; 26: 117−24.

[24] Miroliaei M, Ranjbar B, Naderi-Manesh H, Nemat-Gorgani M. Thermal denaturation of yeast alcohol

dehydrogenase and protection of secondary and tertiary structural changes by sugars: CD and fluorescence studies. Enzyme Microb Technol. 2007; 40: 896−901.

[25] Cheriot SC, Billaud C, Nicolas J. Use of Experimental Design Methodology To Prepare Maillard Reaction Products from Glucose and Cysteine Inhibitors of Polyphenol Oxidase from Eggplant (*Solanum melongena*). J Agric Food Chem. 2006; 54: 5120−6.

[26] Sampedro JG, Uribe S. Trehalose-enzyme interactions result in structure stabilization and activity inhibition. The role of viscosity. Mol Cell Biochem. 2004; 256: 319−27.

[27] López-Dıez EC, Bone S. The interaction of trypsin with trehalose: an investigation of protein preservation mechanisms. Biochim Biophys Acta (BBA) - General Subjects. 2004; 1673:139−48.

[28] Lavelli V, Vantaggi C. Rate of Antioxidant Degradation and Color Variations in Dehydrated Apples as Related to Water Activity. J Agric Food Chem. 2009; 57: 4733−8.

[29] Galmarini MV, Chirife J, Zamora MC, Pérez A. Determination and correlation of the water activity of unsaturated, supersaturated and saturated trehalose solutions. LWT - Food Sci Technol. 2008; 41: 628−31.