

## Dual Effects of Whey Protein Isolates on the Inhibition of Enzymatic Browning and Clarification of Apple Juice

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### Abstract

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The inhibition was studied of enzymatic browning occurring in apple juice by whey protein isolates as compared to ascorbic acid and L-cysteine. A further comparison of different filter-aid pretreatments, such as whey protein isolates (WPI), pectinase and whey protein isolates – pectinase pretreatments, alone and combined with ultrafiltration was also made. The results indicated that WPI demonstrated a concentration-dependent inhibitory activity on polyphenoloxidase (PPO). At lower concentrations (0.005–0.01 g/l), the suppression effect of WPI on PPO activity was higher than that of both ascorbic acid and L-cysteine. WPI exhibited intermediate inhibition on PPO activity at the concentrations of 0.01–0.1 g/l, as compared to ascorbic acid and L-cysteine. The comparison of different clarification treatments suggested that WPI acted more effectively on clarification than pectinase. In addition, combined WPI-pectinase pretreatment significantly increased the clarity of apple juice, indicating a synergistic effect between WPI and pectinase. The subsequent ultrafiltration after WPI and pectinase pretreatments alone or combined could further improve the clarity and colour and reduce the turbidity of clear apple juice with non-significant influence on its typical characteristics.

**Keywords:** apple polyphenoloxidase(PPO); whey proteins; browning; anti-browning agent; clear apple juice

Freshly pressed apple juice is cloudy. The particles in the suspension tend to settle during storage and influence the stability of apple juice. Therefore, fresh apple juice needs to be clarified for obtaining a bright and clear product. Traditionally, filter aids including gelatin and bentonite are used to adsorb and/or coagulate a variety of active haze compounds (OSZMIANŃSKI & WOJDYŁO 2007; QIU *et al.* 2007). Then the process operation such as centrifugation or filtration is applied to remove the particles, improving the product stability and thus its commercial acceptability (GÖKMEN *et al.* 2001). However, this processing is not only costly and laborious but it also adversely influences the sensory and nutritional quality of the product (VELEIRNHO *et al.* 2009). During the last decades, the development of membrane technology such as ultrafiltration (UF) and microfiltration to substitute the conventional

separation techniques has enabled the industrialisation of the whole production, leading to lower the labour requirements and achieve more simplified processing (PADILLA & MCLELLAN 1989). Unfortunately, the performance of the membrane separation is greatly affected by the reducing permeate flux with time due to the membrane fouling (ARAYA-FARIAS *et al.* 2008). Therefore, pretreatments prior to membrane filtration such as those with enzyme, gelatin, bentonite, polyvinylpyrrolidone, and activated carbon have been developed to improve the membrane properties (YOUN *et al.* 2004).

Enzymatic browning is another obstacle encountered in the apple juice industry. Browning impairs the quality of apple juice and thus reduces the market value of the product (QUEIROZ *et al.* 2011). Apple juices are highly susceptible to this deterioration due

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to the very active apple polyphenoloxidase (PPO; EC 1.10.3.1), which rapidly oxidises *o*-diphenols into *o*-quinones, resulting eventually in the formation of browning pigments by a non-enzymatic pathway (SCHULBACH *et al.* 2013). Extensive research has been carried out to explore the inhibitory activities of different anti-browning agents. According to the inhibition mechanisms, anti-browning agents can be categorised as: reducing agents, PPO inhibitors, chelating and complexing agents, and modified atmosphere packaging (ÖZOĞLU & BAYINDIRH 2002). However, very few anti-browning agents are practically used in the food industry due to the concerns over off-flavour and odours, food safety, economic feasibility (LUO *et al.* 2011). Therefore, there is a growing interest to find safe, cheap, and abundant sources of browning inhibitors to increase the stability of food that is susceptible to browning.

Whey protein isolates (WPI) obtained from the dairy industry are natural proteins that have been commonly applied in the food industry (TOMCZYŃSKA-MLEKO 2013). WPI has a pI of 5.1 (XU *et al.* 2013). The surface of the protein molecules is positively charged at the regular apple juice pH 3.0–4.0 (WAKAYAMA 1987). Therefore, the positively charged WPI can react with the negatively charged pectins and polyphenols, which are haze-active components in the cloudy apple juice, thereby causing suspended active haze precursors to condense with one another and settle down. Meanwhile, the viscosity of juice will be decreased, and subsequent UF operation can be utilised to obtain a crystal-clear product. Importantly, PÉREZ-GAGO *et al.* (2003, 2005, 2006) reported that the enzymatic browning of fresh-cut apples was effectively inhibited when coated with whey proteins. They suggest the anti-browning mechanisms of whey proteins are to be attributed to the antioxidation of amino acids (e.g. cysteine) and/or the higher oxygen barrier that the proteins exert. As discussed above, WPI might work as a promising browning inhibitor as well as a clarifying agent in apple juice production. Therefore, the scope of this work was to determine the dual efficacy of WPI as a natural potent anti-browning and aid-clarification agent in apple juice.

## MATERIAL AND METHODS

Apples (cv. Fuji) were purchased at a local market and stored at 4°C until processed. L-cysteine (Cys) and sodium phosphate mono- and dibasic were purchased from Lanji Technology Development Co., Ltd. (Shanghai, China). Ascorbic acid (AA), catechol, and

Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, USA). WPI were purchased from Davisco (St. Le Sueur, USA) and used without further purification. The protein contents of WPI was 97.6% wt. The major protein components of WPI were 55–61%  $\beta$ -lactoglobulin, 19–22%  $\alpha$ -lactoalbumin, and 6–8% bovine serum albumin. EDTA and sodium azide were obtained from Zhengzhou Painsi Chemical Reagent Co., Ltd. (Zhengzhou, China).  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was obtained from Tianli Chemical Reagent Co., Ltd. (Tianjin, China). The copper standard, spectroscopical grade, was purchased from the Analysis and Test Center of National Nonferrous Metals and Electronic Materials (Beijing, China). All the other chemicals mentioned above were of analytical grade.

**Anti-browning treatment.** Apples (4 kg) were washed, peeled, and kept in 2mM phosphate buffer solution (pH 4.0) before being crushed using a Thermomix (Vorwerk, Wuppertal, Germany) laboratory mill. Prior to pressing, WPI, AA, and Cys solutions were prepared by dissolving in 2mM phosphate buffer solution. Then these anti-browning agent solutions of different concentrations (0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 g/l of the apple mash) were added to the apple mash to control the juice browning occurring in the pressing. The apple mash was then pressed using a laboratory screw juice extractor (WF-A2000; Zhejiang Yongkang Tiange Electric Co., Ltd., Zhejiang, China) for 10 min with a yield of 750 ml/kg and was filtered through sterilised nylon cloth. The juice was obtained by centrifugation at 4500 rpm for 10 minutes. After juicing, the samples were analysed to investigate the anti-browning properties of different agents during pressing. The juice sample without any addition of anti-browning agents was taken as control.

**Measurement of copper-chelating capacity of WPI in apple juice modal system.** The capacity of WPI for binding copper ions was evaluated according to the method of CHEN *et al.* (2010) with some modifications. Dialysis bag (molecular mass cut off 2500 Da; Spectrum Laboratories Inc., Rancho Dominguez, USA) was cleaned by heating it twice at 80°C for 30 min in a 1mM EDTA and 2% sodium bicarbonate solution. The bag was then rinsed with deionised water and stored at 5°C in a 0.1% sodium azide solution. WPI solutions with desired concentrations were prepared using 2mM phosphate buffer at pH 4.0. One aliquot of 2.0 ml WPI solution was added into the dialysis bag, equilibrated for 30 min at room temperature in 99 ml of apple juice at pH 4.0, and constantly stirred in a beaker. One millilitre of a 130  $\mu\text{g}/\text{ml}$   $\text{Cu}^{2+}$  solution (prepared from  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

in 2mM phosphate buffer solution at pH 4.0) was pipetted into the beaker, the final concentration of copper ions outside the dialysis bag being 13 µg/ml. After equilibrating for 24 h, the concentration of Cu<sup>2+</sup> outside the dialysis bag was measured by flame atomic absorption spectrophotometer (ICE-3500; Thermo Fisher Scientific, Cambridge, UK) equipped with deuterium background correction, and a Cu hollow-cathode lamp as the radiation source was used in the absorbance measurements. All measurements were carried out in air/acetylene flame. The operating parameters for the working elements were set as recommended by the manufacturer. The instrumental and operating conditions were as follows: wavelength 324.8nm; band pass 0.5 nm; lamp current 6 mA; fuel flow rate 1.1 ml/minute. The standard stock solution (1000 mg/ l) of copper was prepared from copper standard (GSB 04-1725-2004).

**Comparison of influence of pretreatments alone and combined with UF on the juice clarification and its qualities.** In our preliminary experiments, we found that WPI significantly promoted the clarity of apple juice. The current study was conducted to compare the WPI treatment, conventional enzymatic pretreatment and the combination of enzymatic and WPI treatments. Also, the combinations of different pretreatments with UF were evaluated for apple juice clarification and its characteristics. These treatments are shown in Figure 1. 0.68% w/v WPI were added into

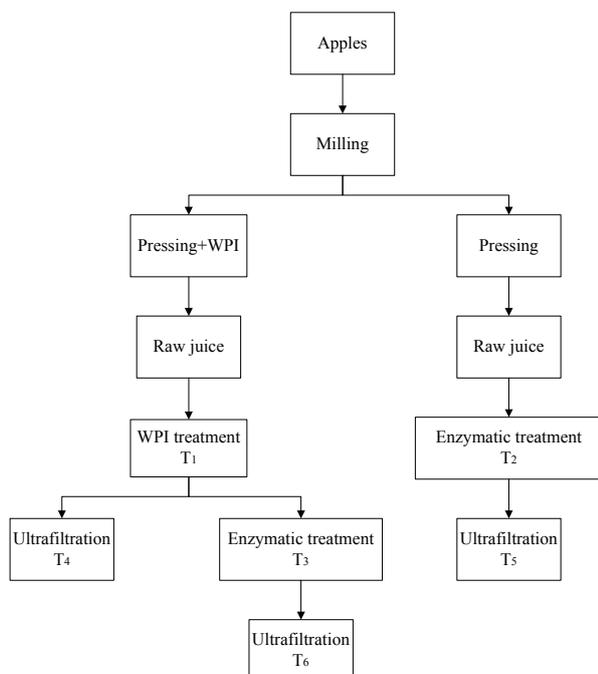


Figure 1. Combination of different pretreatments with UF for apple juice clarification

the apple mash before juicing to prevent browning during pressing. The juice obtained from pressing was heated at 90°C for 2 min and quickly cooled down to 60°C. Then the juice was adjusted to pH 3.0 and incubated for 60 min ( $T_1$ ). The enzymatic treatment was carried out with 0.2 ml/100 ml of Pectinex 100L (Novo Nordisk A/S, Bagsvaerd, Denmark) at 50°C for 60 min ( $T_2$ ), as suggested by OSZMIAŃSKI and WOJDUŁO (2007). In the case of the combination of enzymatic and WPI treatments, WPI treatment was done before enzymatic pretreatments ( $T_3$ ). In the UF experiments, the apple juice was treated with a laboratory scale membrane filtration unit (Mini-pellicon; Millipore Co., Temecula, USA) at the pressure of 1.5 bar with feed rate of 100 ml/min under the retentate recycling back to the feeding tank ( $T_4$ ,  $T_5$ , and  $T_6$ ). The juice sample without any treatment was taken as control.

**Physical and chemical analysis.** Colour measurements were conducted using the  $L^*a^*b^*$  colour space (CIE LAB space) with Minolta Spectrophotometer CM-5 (Minolta Co., Tokyo, Japan). The sample were measured against a standard white reference plate ( $L^* = 98.78$ ,  $a^* = 0.006$ ,  $b^* = 1.56$ ).

Apple PPO activity was measured taking catechol as the substrate and using the modified method of LUO *et al.* (2011). Briefly, 0.5 ml of sample was mixed with 1 ml 0.2M catechol solution and 2 ml phosphate buffer (pH 6.5), and then the changes in absorbance at 420 nm and at 25°C were measured for 2 min using an UNICO Vis-7200 spectrophotometer (UNICO Instruments Co. Ltd., Shanghai, China). PPO activity was evaluated on the basis of the initial reaction rates. The inhibition of enzyme activity was calculated by the formula:

$$\text{Inhibition (\%)} = \frac{(\text{PPO}_{\text{control}} - \text{PPO}_{\text{treatment}})}{\text{PPO}_{\text{control}}} \times 100$$

The clarity was expressed as the transmittance of clarified apple juice at 625 nm using the UNICO Vis-7200 spectrophotometer.

The turbidity was measured with a portable Turbidimeter (Model 2100P; Hach Company, Loveland, USA), and the results were expressed in nephelometric turbidity units (NTU).

The pH of the juice was determined using a PB-10 pH meter (Sartorius AG, Goettingen, Germany). Total acidity (TA) was measured by titrating 20 ml of the sample with 0.1N NaOH to pH 8.2, and the result was converted into malic acid equivalents.

Total soluble solids (TSS) were determined using a WYT-4 hand-held refractometer (Quanzhou Optical Instruments Co. Ltd., Quanzhou, China).

Total phenolics (TPS) were measured with Folin–Ciocalteu reagent method and referred to as mg/l of gallic acid (SPANOS & WROLSTAD 1990).

**Statistical analysis.** All tests were performed in triplicate samples. In all cases, the comparisons of the means were conducted using Duncan's multiple-range tests. The significance level was set at  $P < 0.05$ . All analyses were performed using SPSS v. 17

## RESULTS AND DISCUSSION

### Effect of anti-browning agents on PPO inhibition.

Enzymatic browning starts from the initial phase of apple juice production due to the very fast enzymatic action of PPO soon after the mechanical breakage of the fruit during the juice extraction procedure (KRAPPENBAUER *et al.* 2006). Therefore, it appears to be mandatory to inhibit PPO activity from the beginning of the product transformation. Currently, heat treatment is the most commonly used hurdle for inactivating enzymes. However, a long lag occurs between pressing and thermal processing, and the late heat treatment cannot suppress the browning during juicing. Therefore, in the present work, anti-browning agents were applied before pressing to prevent enzymatic browning during juicing.

The effects of WPI, AA, and Cys at different concentrations on the PPO activity from Fuji apple are shown in Figure 2. The results indicated that the levels of different agents had clearly different influences on the PPO activity as indicated by PPO inhibition determination. WPI demonstrated a concentration-

dependent inhibition activity. WPI are a kind of natural proteins from dairy products (MIHULOVA *et al.* 2013). Also, WPI have been found to inhibit lipid oxidation because of their antioxidant properties. Moreover, according to PÉREZ-GAGO *et al.* (2006), whey protein-based coatings without the addition of antioxidants were effective in retarding enzymatic browning occurring in Golden Delicious apples. However, despite their antioxidant capacity, they had not been previously tested as browning inhibitors applied to the production of apple juice. Figure 2 shows that WPI had an intermediate inhibitory activity on PPO. A higher suppression effect of WPI on PPO was observed throughout the low concentrations (0.005–0.1 g/l) investigated as compared to AA. Increasing the concentrations of WPI (0.1–10.0 g/l) further reduced PPO activity. And at the concentration of 10.0 g/l, PPO was inactivated by more than 80%. However, WPI in the concentration range of 0.1–10.0 g/l could not reduce PPO activity more effectively than AA and Cys.

Currently, the proposed PPO inhibition mechanisms of some proteins (peptides or amino acids) suggested that proteins could inhibit PPO activity by the antioxidant effects of amino acids (e.g. Cys), and/or chelating the essential copper at the active site of PPO (HE *et al.* 2008). However, ELLAS *et al.* (2005) suggested that amino acids, including Cys, can be buried within the interior of proteins thus limiting their effectiveness due to their lack of accessibility to water, in which PPO is dissolved. As discussed previously, positively charged WPI might

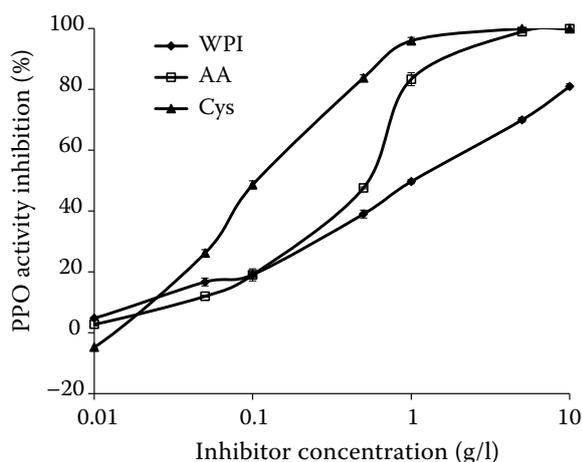


Figure 2. Effects of whey protein isolates (WPI), ascorbic acid (AA), and L-cysteine (Cys) at different concentrations on the PPO activity

Data represent means ( $n = 3$ )  $\pm$  standard deviations; many error bars are within data points

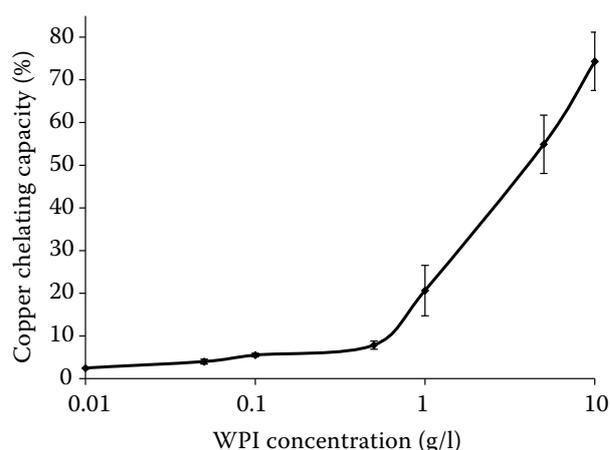


Figure 3. Copper binding percent during incubation of copper ions (13 mg/ml) in model apple juice with various WPI concentrations for 24 h at pH 4.0

Data represent means ( $n = 3$ )  $\pm$  standard deviations; some error bars are within data points

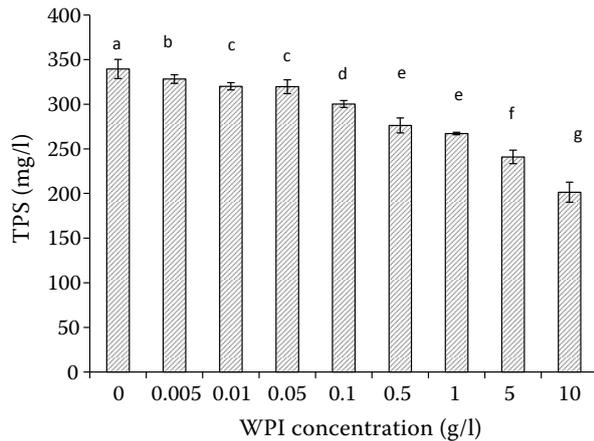


Figure 4. Influence of whey protein isolates (WPI) concentrations on TPS in apple juice at pH 4.0

Data represent means ( $n = 3$ )  $\pm$  standard deviations and different bars with same letters are no statistical difference for least significance difference (*LSD*) test ( $P < 0.05$ ); some error bars are within data points

interact with negatively charged polyphenols to form sediments and reduce free substrates of PPO, thereby leading to the inhibition of PPO activity. In order to understand better how WPI impacted the PPO activity, the chelating capacity of WPI and the influence of WPI on total phenols (TPS) were evaluated in the work as shown in Figures 3 and 4.

Figure 3 shows the influence of the concentration of WPI in model apple juice on their chelating capacity for copper ions. In general, the ability of WPI to chelate copper ions increased with the increasing in concentration of WPI. In a lower range of WPI concentration (0.005–0.5 g/l), a lower chelating capability of copper ions was observed. However, at a higher range of 1 and 10.0 g/l, the chelating capacity of WPI for copper ions was improved substantially. Meanwhile, the reduction of TPS after WPI treatment of apple juice could be also observed in a concentration-dependent manner (Figure 4), confirming that WPI interacted with polyphenols and formed sediments, thus leading to the reduction of polyphenols in the apple juice. These results indicated that the inhibitory property of WPI on PPO activity might be due to their dual abilities to chelate copper at the active site of PPO and reduce polyphenols in apple juice, the substrates for PPO.

AA is frequently used as an anti-browning agent, and the impact of AA on the PPO activity was also studied (Figure 2). AA was more effective than WPI when its concentrations were above 0.1 g/l. However, the anti-browning agent at the concentrations be-

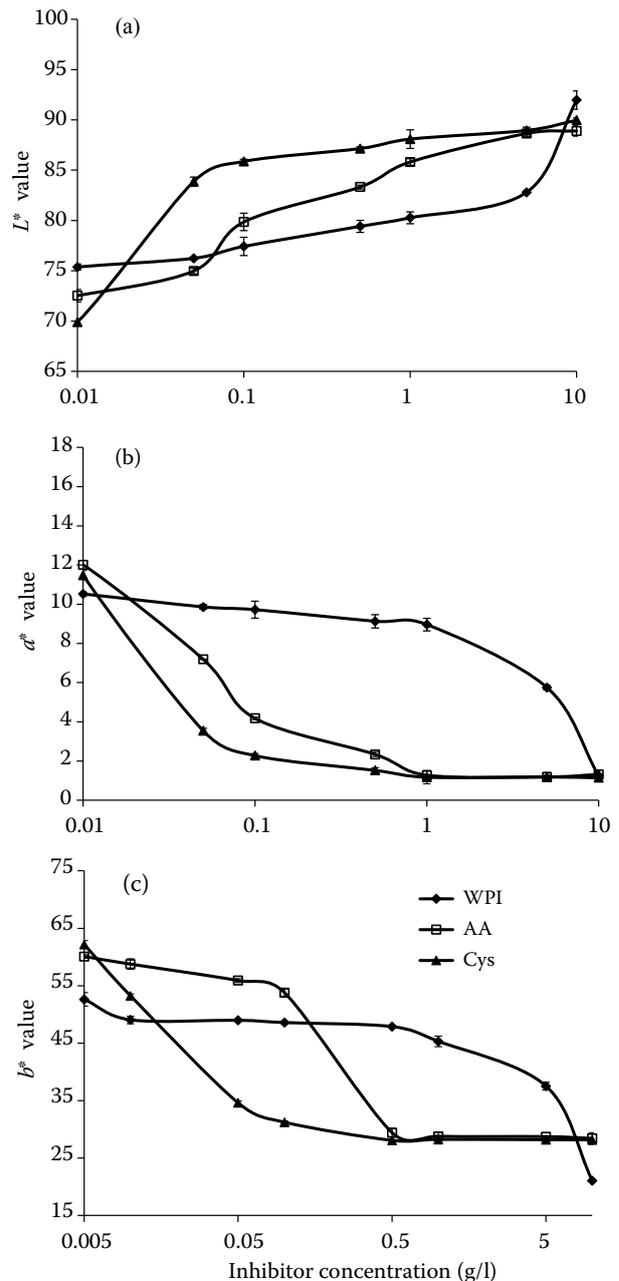


Figure 5. (a)  $L^*$  values of apple juice treated with whey protein isolates (WPI), ascorbic acid (AA), and L-cysteine (Cys) at different concentrations before pressing ( $L^*$  values of apple juice without added WPI, AA, and Cys were  $73.99 \pm 0.64$ ,  $73.5 \pm 0.31$ , and  $73.15 \pm 0.39$ , respectively); (b)  $a^*$  values of apple juice treated with WPI, AA, and Cys at different concentrations before pressing ( $a^*$  values of apple juice without added WPI, AA, and Cys were  $11.19 \pm 0.14$ ,  $11.05 \pm 0.04$ , and  $11.33 \pm 0.12$ , respectively); (c)  $b^*$  values of apple juice treated with WPI, AA and Cys at different concentrations ( $b^*$  values of apple juice without added WPI, AA and Cys were  $53.34 \pm 0.43$ ,  $52.56 \pm 0.62$ , and  $54.12 \pm 0.12$ , respectively)

Data represent means ( $n = 3$ )  $\pm$  standard deviations; many error bars are within data points

tween 0.05 and 1 g/l did not suppress the PPO activity more effectively than did Cys, which was in agreement with previous studies (ÖZOĞLU & BAYINDIRH 2002). A similar inhibitory activity of AA and Cys could be seen at the higher concentrations (> 1.0 g/l). A lower anti-browning activity of AA was also observed at the concentrations below 0.1 g/l as compared to WPI. AA is a highly effective anti-browning agent mainly due to its ability to reduce *o*-quinones to phenols, preventing the formation of pigments. However, the effectiveness of AA in inhibiting browning is greatly lowered if its amount is not sufficient (BILLAUD *et al.* 2003). Lower concentrations of AA can result in complete oxidation of AA, causing the accumulation of *o*-quinones and regeneration of browning compounds (SARPERS 1993). In the study, the low inhibitory effectiveness could be due to AA being consumed at the end of pressing.

The influence of Cys on the enzymatic activity was studied as well (Figure 2), and it showed the highest inhibition of apple PPO at concentrations above 0.05 g/l. Cys retards browning by reacting with *o*-quinones to form stable, colourless compounds. It has been reported that Cys forms a product with catechol, which lowers the PPO activity (DUDLEY & HOTCHKISS 1989). According to the results obtained (Figure 2), Cys appeared to inhibit completely the PPO activity at the concentrations above 1.0 g/l. But such high concentrations caused unfavourable odour and a bleaching effect. Similar phenomena were observed in the cloudy apple juice with added Cys (ÖZOĞLU & BAYINDIRH 2002). It can be also observed in Figure 2 that the inhibitory extent of PPO by Cys was below 0% at low concentrations (< 0.01 g/l). RICHARD-FORGET *et al.* (1991, 1992) proposed that Cys at low critical concentrations could cause excessive production of *o*-quinones, which co-oxidise the Cys-quinone compound, resulting in phenol regeneration with a deep colour formation. In the case of this study, it seems that Cys at lower concentrations (< 0.01 g/l) induced the colour development.

**Colour change.**  $L^*$ ,  $a^*$ , and  $b^*$  values of the apple juice treated with WPI, AA, and Cys at different concentrations before pressing are shown in Figure 5. On the basis of the changes in  $L^*$ ,  $a^*$ , and  $b^*$  (this sentence is not complete). Again, WPI inhibit browning in a concentration-dependent manner. Importantly, the apple juice treated with WPI at 10.0 g/l had the highest  $L^*$  and the lowest  $a^*$  and  $b^*$ , although the inhibition of PPO activity by WPI was 80.95%. The reason for this observation was that WPI interacted with polyphenols, and then reduced the content of soluble polyphenols in the apple juice,

thereby increasing the value  $L^*$  and lowering  $a^*$  and  $b^*$  according to the results shown in Figure 4.

It was also found that the treatments with Cys and AA at a concentration below 0.01 g/l even reduced  $L^*$  and increased  $a^*$  and  $b^*$  as compared to the controls (Figure 5), which indicated that Cys and AA at the concentrations studied promoted browning in the apple juice. The possible reason for the phenomena resides in the fact that the complete oxidation of AA led to the accumulation of quinones and thus the increased browning when AA was at low concentrations as explained previously. With regard to Cys, the data obtained from Figure 5 confirmed the findings above stating that Cys at low concentrations induced a deep colour development. Cys again proved to be the most effective browning inhibitor out of these inhibitors based on  $L^*$ ,  $a^*$ , and  $b^*$  at the concentrations of 0.1–1.0 g/l. However, Cys and AA at high concentrations (above 1.0 g/l) also showed similar suppressive capacity toward browning in the values of  $L^*$ ,  $a^*$ , and  $b^*$ .

**Comparison of different clarification treatments.** The physical and chemical properties of apple juice were measured to compare the efficacy of various treatments, i.e., WPI pretreatment alone ( $T_1$ ), enzymatic pretreatment alone ( $T_2$ ), WPI-enzymatic pretreatment ( $T_3$ ), ultrafiltration after WPI, enzymatic and WPI-enzymatic pretreatment, respectively ( $T_4$ ,  $T_5$ , and  $T_6$ ). As shown in Table 1, juice transmittance was greatly influenced by filter-aid pretreatments.  $T_2$  had a lower clarity compared to  $T_1$ , suggesting pectinase was less effective than WPI in the present work. Interestingly,  $T_3$  revealed the highest transmittance followed by  $T_1$  and  $T_2$ , indicating there was a synergistic effect between WPI and pectinase. The possible reason was attributed to their different clarification mechanisms. As stated earlier, WPI might work by electrostatic attraction to cause particles to stick to one another, making them heavy enough to sink to the bottom by the action of gravity, whereas pectinase could improve juice clarity by decomposing pectins into small molecules and decreasing the viscosity of the apple juice. In this way, the combination of enzymatic and WPI pretreatments could further increase the apple juice clarity. TAJCHAKAVIT *et al.* (2001) also reported that enzymatic treatment, assisted by clarifying agents such as gelatin (proteins) and bentonite, could effectively remove clouds compounds in apple juice. Moreover, a similar trend was observed with juice turbidity after different pretreatments the effect being in the order of  $T_3 < T_1 < T_2$ , thus confirming

Table 1. Effects of different treatments on physical and chemical properties of apple juice

Treatments	Transmittance (%)	Turbidity (NTU)	$L^*$	$a^*$	$b^*$	TPS (mg/l)	TA	TSS
							(g/100g)	
Control	17.13 ± 0.70 <sup>e</sup>	599.41 ± 0.46 <sup>a</sup>	65.49 ± 0.25 <sup>f</sup>	8.81 ± 0.03 <sup>a</sup>	45.43 ± 0.04 <sup>a</sup>	332.41 ± 7.64 <sup>a</sup>	0.21 ± 0.03 <sup>a</sup>	14.06 ± 1.47 <sup>a</sup>
T <sub>1</sub>	84.50 ± 0.40 <sup>c</sup>	38.20 ± 0.21 <sup>c</sup>	92.90 ± 0.10 <sup>c</sup>	-1.55 ± 0.06 <sup>c</sup>	10.22 ± 0.19 <sup>c</sup>	225.55 ± 0.70 <sup>c</sup>	0.20 ± 0.01 <sup>a</sup>	14.05 ± 1.44 <sup>a</sup>
T <sub>2</sub>	79.63 ± 0.55 <sup>d</sup>	45.40 ± 1.00 <sup>b</sup>	85.56 ± 0.07 <sup>e</sup>	-0.96 ± 0.11 <sup>b</sup>	36.17 ± 0.54 <sup>b</sup>	298.52 ± 6.75 <sup>b</sup>	0.22 ± 0.02 <sup>a</sup>	13.77 ± 0.27 <sup>a</sup>
T <sub>3</sub>	92.46 ± 1.05 <sup>b</sup>	14.43 ± 0.81 <sup>d</sup>	98.32 ± 0.01 <sup>b</sup>	-1.53 ± 0.03 <sup>c</sup>	10.36 ± 0.24 <sup>c</sup>	204.90 ± 4.35 <sup>d</sup>	0.21 ± 0.03 <sup>a</sup>	13.61 ± 0.15 <sup>a</sup>
T <sub>4</sub>	98.90 ± 0.60 <sup>a</sup>	2.22 ± 0.10 <sup>e</sup>	99.57 ± 0.10 <sup>a</sup>	-2.08 ± 0.06 <sup>d</sup>	5.39 ± 0.01 <sup>e</sup>	188.87 ± 1.34 <sup>e</sup>	0.21 ± 0.00 <sup>a</sup>	13.85 ± 0.08 <sup>a</sup>
T <sub>5</sub>	98.33 ± 0.15 <sup>a</sup>	1.38 ± 0.03 <sup>e</sup>	90.25 ± 0.17 <sup>d</sup>	-2.87 ± 0.01 <sup>f</sup>	7.88 ± 0.01 <sup>d</sup>	259.49 ± 1.01 <sup>c</sup>	0.21 ± 0.02 <sup>a</sup>	13.75 ± 0.17 <sup>a</sup>
T <sub>6</sub>	99.11 ± 0.41 <sup>a</sup>	0.41 ± 0.01 <sup>f</sup>	99.60 ± 0.13 <sup>a</sup>	-2.40 ± 0.01 <sup>e</sup>	4.36 ± 0.01 <sup>f</sup>	176.45 ± 3.04 <sup>f</sup>	0.20 ± 0.02 <sup>a</sup>	13.50 ± 0.29 <sup>a</sup>

Values are means ( $n = 3$ ) ± standard deviation; the same letters in the same column present no statistical difference at  $P < 0.05$

the incorporation of WPI + pectinase to be the best pretreatment in this work.

That what remained after all these pretreatments, including WPI treatment, enzyme treatment and their combination, was transparent but not clear juice (Table 1). As a result, subsequent UF operation was needed to produce clear juice. In recent years, the conventional clarification process was been replaced by the use of UF. However, as mentioned above, UF can not be used alone to clarify fruit juice because of the decrease of reflux and failure in removing active haze precursors. It can clearly be seen in Table 1 that the clarity of the apple juice was significantly improved by UF after all the pretreatments, indicating the UF was very effective in clarifying the apple juice when it was combined with filter-aid pretreatments. Our results are also in agreement with the observations made by YOUN *et al.* (2004) and ONSEKIZOGLU *et al.* (2010).

As shown with pretreated juice, the properties of the apple juice were remarkably improved by the treatment with clarifying agents with  $L^*$  increasing and  $a^*$  and  $b^*$  decreasing in comparison to the control. WPI treatment resulted in higher colour lightness and lower redness and yellowness compared to enzymatic treatment. Also, the colour of the apple juice was slightly improved by WPI + pectinase treatment compared with WPI alone although not significantly, suggesting that WPI was the main factor for the juice colour improvement. The impact of protein-aid treatment on the improvement of colour values was also reported by some other authors (ALONSO-SALCES *et al.* 2005; OSZMIAŃSKI & WOJDYŁO 2007). In addition, there was a further improvement of the juice colour after a combined application of filter-aid pretreatments and UF. It is well documented that the juice colour is greatly dependent on the PPO activity (PADILLA & MCLELLAM 1989). The findings given

above suggested that WPI could somehow inhibit PPO activity. Also, Table 1 shows that WPI significantly reduced TPS ( $P < 0.05$ ), which could help explain why the incorporation of WPI either alone or combined with other treatments could greatly improve the juice colour. With respect to the reduction of phenolic compounds by protein-aid treatment, GÖKMEN *et al.* (2001) and OSZMIAŃSKI and WOJDYŁO (2007) also demonstrated that the traditional clarification using gelatin could reduce the content of phenols. These findings were in agreement with our study. Enzyme treatment also significantly reduced TPS, which is in agreement with the findings by MARKOWSKI *et al.* (2009). In addition, a decrease of TPS in the apple juice treated with UF also explained the colour improvement after UF.

Total acidity and soluble solids were not changed significantly by the pretreatments and UF processing, indicating that the typical nutritional properties of apple juice were well preserved during clarification

## CONCLUSIONS

WPI had dual function in apple juice anti-browning activity and clarification. The obtained results show that WPI can inhibit PPO activity in a concentration-dependent manner. The mechanisms by which WPI inhibited PPO activity were probably due to their chelating capacity for copper ions at the active site of PPO, and the reduction of polyphenols. The comparison of WPI, pectinase, and their combination showed that WPI pretreatment was better than enzymatic pretreatment, and that there was a synergistic effect on apple juice clarification between WPI and enzyme treatments. UF after WPI and pectinase pretreatments both singly and combined could further improve the clarity and colour, and reduce the turbidity of apple juice with non-significant influ-

ence on its nutritional characteristics. In this way, WPI, natural abundant proteins, can be proposed as a potential anti-browning agent and an alternative aid for apple juice clarification.

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