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Inhibition of apricot polyphenol oxidase by combinations of plant proteases and ascorbic acid

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ABSTRACT

The present research investigates the long term inhibition of enzymatic browning by inactivating the polyphenol oxidase (PPO) of apricot, using combinations of plant proteases and ascorbic acid (AA). The selected proteases were able to inactivate PPO at pH 4.5, with the degree of inactivation proportional to incubation time and protease concentration. Papain was the most effective protease, with 50 µg completely inactivating PPO in less than one hour. AA prevented browning reactions that occur before or during PPO inactivation by protease. The combinations of AA/proteases were highly effective *in vitro,* where 2 mM AA/500 µg proteases inhibited PPO activity completely over 24 h. The combination of AA/proteases was also effective *in vivo*, as treated apricot purees preserved their color ($p < 0.0001$, compared to untreated samples after 10 days of storage). The results demonstrate that AA/proteases combinations constitute a promising practical anti-browning method with feasible application in the food industry.

1. Introduction

Apricot (*Prunus armeniaca* L.) is a globally consumed fruit. Apricot is mainly appreciated for its specific color and flavor (Melgarejo et al., 2014). However, apricot as a climacteric fruit undergoes fast post-harvest maturation (Supplementary A), making it highly perishable and sensitive to handling. Physical stress such as bruising, compressing, and cutting can disorder the pericarp cells, triggering undesirable enzymatic browning. Browning reactions affect the appearance, flavor, and nutritive value of apricot and many other fruits (apple, pear, banana, peach, etc.), which limits their shelf life. Furthermore, this phenomenon is among the biggest problems in fruit conservation and processing. It has been reported that enzymatic browning is responsible for more than 50% of fruit industry losses (Whitaker & Lee, 1995). Browning reactions in fruits are mainly triggered by polyphenol oxidases (PPOs). These enzymes oxidize phenolic compounds to mainly colorless compounds known as quinones. However, once this reaction has taken place, the reactive quinones can polymerize spontaneously and produce melanins (brown pigments) which results in browning (Vomas-Vigyazo, 1981; Whitaker & Lee, 1995).

PPOs are copper-containing enzymes belonging to the group of oxidoreductases. They oxidize monophenols and diphenols in the presence of molecular oxygen (Whitaker & Lee, 1995). PPOs are widely distributed in nature and can be found in the majority of plants, animals and microorganisms (Mayer, 2006). However, PPO properties vary widely between species. Plant PPOs are generally expressed in a latent (enzymatically inactive) form, which contains a catalytically active domain shielded by a C-terminal domain. The latent form can be activated by limited proteolysis, acidic pH, fatty acids, or detergents (Mayer, 2006). Recently, it has been reported that the latent form can spontaneously activate during the first weeks of storage, generating an active form with a molecular weight of 38 kDa (Derardja, Pretzler, Kampatsikas, Barkat, & Rompel, 2017; Kampatsikas, Bijelic, Pretzler, & Rompel, 2019). Apricot polyphenol oxidase had been studied during the last years; the enzyme was purified in its latent form (63 kDa), exhibiting a weak monophenolase activity and a strong diphenolase activity at the optimum conditions of pH 4.5 and 30 °C (Derardja et al., 2017).

Due to their direct connection to enzymatic browning, PPOs have been the subject of extensive research carried out in the last few

Abbreviations: AA, ascorbic acid; CI, (time of) complete inhibition; PPO, polyphenol oxidase; P, protease preparation; *p*, probability; SE, standard error

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decades by food scientists and technologists. The research mainly aims to control enzymatic browning by inhibiting PPO (McEvily, Iyengar, & Otwell, 1992). Enzymatic browning can be prevented by the inactivation of the enzyme, exclusion or removal of the substrates (O_2 and phenols), moving the conditions away from the optimum for the enzyme by lowering or increasing the pH and temperature, and by adding compounds that inhibit PPO or prevent melanin formation (Whitaker & Lee, 1995). Up to date, most of the methods that have been developed focused mainly on the last approach, and hundreds of compounds have been tested as enzymatic browning inhibitors (McEvily et al., 1992; Mesquita & Queiroz, 2013). Several chemicals proved their efficiency in controlling enzymatic browning, especially reducing compounds like ascorbic acid (AA) and sulphites (Vomas-Vigyazo, 1981; McEvily et al., 1992). These two compounds are the most used browning inhibitors in the food industry. However, they are necessarily consumed during the process of inhibition and thus, provide only temporary protection against browning (Vámos-Vigyázó, 1981). Furthermore, the use of sulphites and their derivates is restricted nowadays, due to their negative effect on consumer health (Whitaker & Lee, 1995; FDA, 2000). This prompted a refocusing of the research on enzymatic browning inhibition, towards more healthy and safe anti-browning agents like ascorbic acid. AA (vitamin C) is considered one of the major compounds used in the food industry, owing to its conservatory effects mainly as the most effective and least toxic antioxidant. AA is an essential nutrient for humans, as we cannot synthesize vitamin C and must acquire it from our diet. However, AA is rapidly oxidized and significant losses are observed during food storage (Mellidou, Koukounaras, Chatzopoulou, Kostas, & Kanellis, 2017).

The approach of preventing enzymatic browning by inactivating PPOs had received less attention. Heat inactivation is the most common method used to inactivate PPOs in the food industry (Marshall, Kim, & Wei, 2000). Most PPOs can be inactivated by subjecting the fruits to heat treatments (85–100 °C for 1 to 10 min). However, heat treatments have been found to significantly affect the nutritional and sensorial qualities of the fruit by reducing the content or the bioavailability of some bioactive compounds (phenolic compounds, carotenoids and vitamins) and inducing several chemical and physical changes that impair the flavor, the taste and the firmness of the fruit (Rawson et al., 2011).

In the last decades, interest in plant proteases has increased rapidly. Proteases are the commercially most important enzymes due to their multiple applications in the food, pharmaceutical and detergent industries. Protease preparations from plant have great potential in the food industry; they are used in many applications such as cheese-making, meat softening, brewing and in the beverage industries. They can also be used to improve the nutritional characteristics by retarding deterioration, preventing undesired interactions, modifying functional properties such as solubility, foaming, coagulation, and emulsifying capacities (González-Rábade, Badillo-Corona, Aranda-Barradas, & Oliver-Salvador, 2011). The majority of plant-derived proteases have been classified as cysteine proteases, and the most widely utilized plant proteases are papain, bromelain, and ficin, extracted from *Carica papaya*, *Ananas comosus* and *Ficus carica*, respectively (González-Rábade et al., 2011). Recently, calotropain, a cysteine protease extracted from *Calotropis procera*, has also drawn attention due to its high proteolytic activity (Anusha, Singh, & Bindhu, 2014). These proteolytic enzymes are very attractive since they can be active over a wide range of temperature and pH (González-Rábade et al., 2011).

In theory, PPOs can also be inactivated by digestion if subjected to strong proteolytic enzymes. Thereby, the degradation of PPOs by proteases will induce their inactivation. Ficin, papain, and bromelain have been reported to be effective in controlling enzymatic browning (McEvily et al., 1992; Lozano-De-Gonzalez, Barrett, Wrolstad, & Durst, 1993). In addition, several proteases (ficin, actinidin, papain, and bromelain) were tested on apple slices by Labuza, Lillemo, and Taoukis (1992), and the proteases proved their effectiveness as enzymatic browning inhibitors. Albeit, Lozano-De-Gonzalez et al. (1993) tested the effect of multiple fractions of pineapple juice as browning inhibitors on apple slices. They reported a low enzymatic browning inhibition with the protein fractions compared to the non-protein fractions (that contain low molecular compounds) of the juice. However, in those studies, most of the protease preparations were applied without taking into consideration that PPO inactivation with proteases is a time-consuming reaction, and that PPO may still be active during most of the digestion process. Thus, proteolytic digestion needs a certain incubation time to inactivate PPOs. The necessary incubation time depends on the ratio of proteolytic activity which is determined by the protease (group, concentration, specificity, etc) and substrate (PPOs) loading as well as on the reaction conditions (pH, temperature) (González-Rábade et al., 2011).

In this context, the main goal of this research is to evaluate the potential of some plant protease preparations as inhibitors of enzymatic browning by studying their ability to inactivate PPO, the main enzyme responsible for enzymatic browning of apricots. In addition, we were also studying the possibility of a combination between protease preparations and AA, where AA is mainly used to inhibit PPO during the time needed by the proteases to completely inactivate PPO.

2. Materials and methods

2.1. Raw materials

Fruits of *Prunus armeniaca* L. cv. Bulida, were harvested from a local farm in the M'sila region of Algeria at commercial maturity in June 2017. The fruits were rinsed with tap water, deseeded, frozen in liquid nitrogen, immediately broken into small pieces with mortar and pestle, lyophilized, and stored at −25 °C until use. Moreover, latex of *Calotropis procera* from Adrar, Algeria, and latex of *Ficus carica* from Bordj Bou Arreridj, Algeria, were collected during March and July 2017, respectively, put in clean tubes and stored at −25 °C. Lyophilized powders of papain from the latex of *Carica papaya* and bromelain from the stem of *Ananas comosus* were purchased from Roth (Vienna, Austria).

2.2. Extraction and purification of PPO

Apricot PPO was extracted and purified (Fig. 1A) as described previously (Derardja et al., 2017). Lyophilized apricots (100 g) were extracted several times with cold acetone until a white powder was obtained. The resulting acetone powder was homogenized in 1 L of sodium phosphate buffer (0.1 M) at pH 6.8 containing 30 mM sodium ascorbate, 1% (w/v) polyvinylpolypyrrolidone, 0.5% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The homogenate was stirred, and then centrifuged at 30,000 × *g* for 30 min at 4 °C. The supernatant was subjected to $(NH_4)_2SO_4$ precipitation (85% saturation). The precipitate was dissolved in 250 ml of 10 mM sodium phosphate buffer (pH 6.8) and dialyzed overnight at 4 °C against Tris-HCl buffer (10 mM, pH 8.0). The dialyzed solution was applied to an ÄKTA fast protein liquid chromatography system (FPLC). The protein solution was purified over an anion exchange column (Q-Sepharose FF, 20 ml) followed by a cation exchange step (Mono S HR 5/50 GL). The active fractions (pure PPO) were pooled, washed with 10 mM Tris-HCl buffer pH 8.0 and concentrated by ultrafiltration to be used in further experiments.

2.3. Proteases extraction

The extraction of proteases from the latex of *Calotropis procera* and *Ficus carica* was done according to the method of Nath and Dutta (1991) with slight modifications. To one volume of latex, two volumes of distilled water were added and the mixture was kept overnight in a deep-freezer at −25 °C. The floating gum was removed, and the latex solution was centrifuged at 10,000 × *g* for 10 min at 4 °C. The

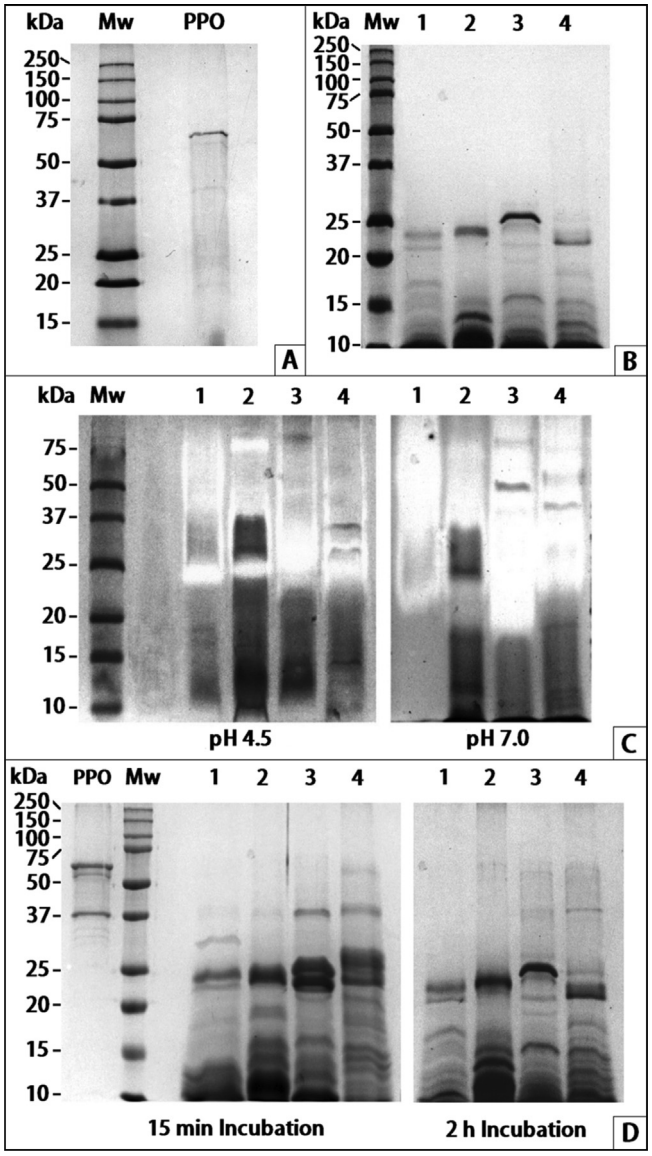

Fig. 1. Electrophoresis analysis. (A) SDS-PAGE of PPO purified from apricot: Mw, molecular weight markers; PPO, purified apricot polyphenol oxidase. (B) SDS-PAGE of protease preparations: Mw, molecular weight markers; lane 1, papain; lane 2, calotropain; lane 3, ficin; lane 4, bromelain. (C) Zymograms of protease preparations at pH 4.5 and 7.0: Mw, molecular weight markers; lane 1, papain; lane 2, calotropain; lane 3, ficin; lane 4, bromelain. (D) SDS-PAGE of PPO proteolysis by protease preparations after 15 min and 2 h of incubation at pH 4.5: PPO, purified apricot polyphenol oxidase ; Mw, molecular weight markers; lane 1, papain + PPO; lane 2, calotropain + PPO; lane 3, ficin + PPO; lane 4, bromelain + PPO.

supernatant was filtered through a Whatman paper No. 1, and was then freeze dried. The lyophilized powder was used as the starting material for the further experiments ("protease preparation").

2.4. Enzyme activity and protein concentration

PPO activity was determined at 25 °C in 200 µl of assay mixture by measuring the increase in absorbance at 410 nm using a microplate reader (Infinite M200, Tecan). The standard reaction mixture consisted of 1 µg of enzyme and 10 mM catechol in 50 mM sodium citrate buffer (pH 4.5). PPO activity (catecholase activity) was determined from the slope of the initial linear part of the experimental curves (absorbance vs. time) and expressed as U/ml. One unit of enzymatic activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol of quinones per minute (1 U = 1 µmol/min). The protein concentration measurement was carried out according to the method of Bradford (1976) using bovine serum albumin as standard.

2.5. Gel electrophoresis

Denaturing SDS-PAGE was performed as described by Laemmli (1970) in a mini gel apparatus (Mini-PROTEAN Tetra Cell, Bio-Rad). PPO (5 µg) and protease preparations (20 µg) were denatured and applied under reducing conditions in 10–15% linear polyacrylamide gradient gels. Gels were stained overnight with Coomassie Brilliant Blue G-250, and the molecular weight was estimated by comparison to molecular weight markers (Precision Plus Protein Standard Dual Color, Bio-Rad).

2.6. Proteases zymography

The development of activity zones (zymography) of the protease preparations was done according to García-Carreño, Dimes, and Haard (1993). The SDS-PAGE was performed as described above with the two sole modifications of omitting the β-mercaptoethanol in the loading buffer and the samples were not heated before loading onto the gels. 20 µg of protease preparations were applied to the gels. After electrophoresis, gels were soaked in a 2.5% Triton X-100 solution for 30 min at 4 °C to remove SDS. Then the gels were immersed in 50 ml of 2% casein in 50 mM citrate buffer, pH 4.5 or 50 mM Tris-HCl buffer, pH 7.0 for 30 min at 4 °C, in order to allow the substrate to diffuse into the gel, while maintaining low enzymatic activity. Then the temperature was raised to 25 °C and the gels were incubated for 90 min to allow for the digestion of the protein substrate. After the incubation, gels were washed with distilled water, fixed and stained overnight with Coomassie Brilliant Blue G-250.

2.7. PPO proteolysis

PPO hydrolysis with proteases was monitored by SDS-PAGE. 5 µg of PPO was added to 20 µg of protease preparation in 50 mM of sodium citrate buffer pH 4.5. The mixture was incubated for two different time periods (15 min and 2 h). Then the reducing buffer was added and the mixture was heated and loaded on the gel. A control assay without protease preparations consisting of 5 µg of PPO in 50 mM of sodium citrate buffer pH 4.5 was also loaded. SDS-PAGE was carried out as described above.

2.8. Inhibition with ascorbic acid (AA)

The effect of AA on PPO activity was studied with various concentrations (0.1, 0.2, 0.5, 1, 2, 5 and 10 mM) of AA, and PPO activity was determined in activity Units (catecholase activity) as described above. In case of complete PPO inhibition by AA the reaction was monitored for 24 h, unless PPO activity was recovered earlier. Enzymatic activity is presented in the form of percent residual activity relative to the PPO activity of the control mixture without AA (100%).

2.9. Inhibition with proteases

To study the effect of the selected protease preparations (papain, calotropain, ficin, and bromelain) on PPO activity, PPO (1 µg) was incubated in 50 mM sodium citrate buffer pH 4.5 with different amounts of protease preparations (50, 100, 200 and 500 µg). In order to assess the effect of the proteases on PPO at pH 7.0 (optimum pH generally reported for the selected proteases), 1 µg of PPO was incubated with 100 µg of protease preparations in 50 mM Tris-HCl buffer, pH 7.0. The different mixtures were incubated for different incubation times (0, 2, 5, 10, 20, 30 min, 1, 2, 4, 8, 16 and 24 h), before adding the substrate

(catechol) and measuring PPO activity as described above. The enzymatic activity of the control mixture at pH 4.5 without proteases was defined as 100%.

2.10. Combined treatments with AA and proteases (AA/P)

According to the results of PPO inhibition with AA, the concentrations (0.1, 0.5, 1 and 2 mM) of AA, which did not give a complete inhibition (CI) of PPO over 24 h were selected for combination with proteases. The combinations of AA and proteases (AA/P) were mixed with 1 μg of PPO (16 pmol), and the enzymatic activity was determined as described earlier at pH 4.5. The reaction was monitored over 24 h to check for a recovery of PPO activity. The enzymatic activity of the control mixture at pH 4.5 without inhibitors was defined as 100%.

2.11. Application on apricot puree and color measurement

The effect of treatment with AA/P combinations on apricot puree was studied using 100 and 500 mg of AA and 10, 50 and 100 mg of protease preparations per 100 g of fresh apricots. The AA/P combinations were chosen based on the results of PPO inhibition *in vitro*, where the amount of PPO present in fresh apricots was estimated from the results of the apricot PPO purification (Derardja et al., 2017). The AA/P combinations were prepared in 1 ml of distilled water. The pH and the total soluble solid (TSS) content of the puree samples were 4.38 ± 0.33 and 14.1 ± 0.3 °Brix, respectively.

Apricots were cleaned and deseeded. Then, 100 g were introduced into a Hand Blender Beaker with 1 ml of the freshly prepared AA/P solution. The mixture was blended for 2 min. A control puree was prepared without inhibitors. In addition, to allow for a better comparison, apricot puree was also treated only with AA (50, 100, 500 and 1000 mg), without added protease. After homogenization, apricot purees were packaged in 250 ml plastic boxes made of polypropylene. The boxes were stored at 4 °C in darkness until analysis, for up to 10 days. Apricot puree color was measured immediately after homogenization to determine the initial color (0 min). Afterwards, the color of the apricot puree was determined after 5, 10, 30 min; 1, 2, 4, 8, 16 h; 1, 2, 5 and 10 days. The surface color of treated and untreated purees was measured by a computer vision system (CVS) and expressed as CIELAB Tristimulus coordinates L^*, a^*, b^*.

The computer vision system consisted of a standardized lighting system (photo shooting box) with three LED spotlights (6500 K daylight, 6 W), a Canon EOS-1200D digital camera (18 megapixel resolution and 3x 18–55 mm f/3.5–5.6 Zoom Lens), and a computer with Adobe Photoshop CS6 software (Adobe Systems Inc., USA).

For each assay, 3 g of apricot puree were smeared on a labeled transparent plastic square (70 × 70 × 0.2 mm) and then placed inside the photo shooting box on a white background. All photos were taken in manual mode (1/80, F5.6, ISO400). The measuring procedures of the color were as described by Zhou, Ling, Zheng, Zhang, and Wang (2015). The color values: lightness (L), redness-greenness (a) and yellowness-blueness (b) were obtained from the histogram of the menu bar by using LAB color mode in Adobe Photoshop. The rectangular marquee tool in the main menu was used to select the sample area. Then the L, a, b values of the selected area were transformed to CIELAB (L^*, a^*, b^*) values using the equations (Supplementary B):

$$L^* = \frac{L}{2.5}$$

$$a^* = \frac{240a}{255} - 120$$

$$b^* = \frac{240b}{255} - 120$$

The total color difference, ΔE was calculated according to the following equation:

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

L^*, a^*, and b^* are the color values of the samples, while $L_0{}^*$, $a_0{}^*$, and $b_0{}^*$ are the color values of the puree which exhibited minimal browning (puree treated with 1000 mg AA at 0 min).

2.12. Statistical analysis

Each experiment was repeated three times and the data was subjected to an analysis of variance (ANOVA). Significant difference was determined by Tukey's multiple range test ($p \leq 0.05$) using XLSTAT software Version 2009. The data values were expressed as mean ± standard error (SE) (n = 3).

3. Results and discussion

3.1. Gel Electrophoresis, zymography and PPO proteolysis

PPO used in this study was purified in its latent form exhibiting a single band on SDS-PAGE (Fig. 1A). The enzyme had the same characteristics as reported in our previous research (Derardja et al., 2017); pH optimum of 4.5, activation during storage, a molecular weight of 63 kDa for the latent form of PPO and 38 kDa for the active form of PPO.

SDS-PAGE analysis of the protease preparations is shown in Fig. 1B. All extracts showed clear protein bands with molecular weights between 23 and 27 kDa. The molecular weights of the main bands observed on the gel are 23.7, 24.5, 26.5 and 23 kDa for papain, calotropain, ficin and bromelain, respectively. These molecular masses are consistent with the values reported for papain (23 kDa) (Mathew & Juang, 2005), calotropain (25–26 kDa) (Ramos et al., 2013), ficin (27 kDa) (Baeyens-Volant, Matagne, El Mahyaoui, Wattiez, & Azarkan, 2015) and bromelain (24 kDa) (Matagne, Bolle, El Mahyaoui, Baeyens-Volant, & Azarkan, 2017), and fall within the general range of molecular weights reported for cysteine proteases (21–30 kDa) (Grzonka, Kasprzykowski, & Wiczk, 2007).

The verification of the proteolytic activity of the protease preparations at pH 4.5 and 7.0 using the SDS-substrate gel (zymogram) is shown in Fig. 1C. The staining was performed at pH 4.5 and 7.0 in order to assess the proteolytic activity of the selected proteases at the optimum pH of PPO and proteases, respectively. Clear zones on dark background indicate protease activity. The undigested casein during staining is responsible for the slight darkening of the background (García-Carreño et al., 1993). The zymogram at pH 4.5 showed proteolytic activities in several zones, where all protease lanes contained more than one protein capable of producing a clearing zone. However, the main activity zones had molecular weights ranging from 22 to 27 kDa, which is in accordance to the bands observed on denaturing SDS-PAGE. The clear activity zones expended for the zymogram stained at pH 7.0 and they were more intense for most bands, which made a precise positioning of the bands difficult. This can be explained by the increase of proteolytic activity under these conditions at neutral pH, where papain (Homaei, Sajedi, Sariri, Seyfzadeh, & Stevanato, 2010), calotropain (Freitas et al., 2007), ficin (Devaraj, Kumar, & Prakash, 2008), and bromelain (Harrach et al., 1998) are optimally active. This is also consistent with the observation of casein agglomeration at pH 4.5 (Post, Arnold, Weiss, & Hinrichs, 2012), which may affect their hydrolysis by the protease preparations.

According to these results, we confirmed the presence of active proteases in our extracts. The proteolytic activity was higher at pH 7.0 for most proteases. However, all proteases still exhibit pronounced proteolytic activity at pH 4.5, allowing their use on PPO at acidic pH. Papain (Homaei et al., 2010), ficin (Devaraj et al., 2008), calotropain (Freitas et al., 2007; Anusha et al., 2014), and bromelain (Harrach et al., 1998) had been found to conserve activity at low pH, and they have been successfully used in many applications as e.g. in milk clotting

and meat tenderization (Grzonka et al., 2007).

In order to study PPO proteolysis capability of the protease preparations, 5 µg of PPO were subjected to hydrolysis by protease preparations for 15 min and 2 h followed by display on SDS-PAGE (Fig. 1D). As we reported in our previous work, PPO can be activated during storage, generating an active form with a molecular weight of 38 kDa (Derardja et al., 2017). This can explain the appearance of the active form in the control lane (Fig. 1D), where part of the purified latent PPO was converted to active PPO (PPO proteolysis was performed one week after PPO purification). All four protease preparations were able to hydrolyze PPO, but with different efficiencies. After 15 min of incubation, papain and calotropain preparations were able to hydrolyze PPO almost completely. Latent PPO was completely hydrolyzed, and only hardly visible slight bands of active PPO are left. However, with ficin and bromelain PPO was only partially digested after 15 min, where the bands of active PPO are still obvious and almost as strong as the respective bands of the control lane. After 2 h of incubation, the protease preparations had completely hydrolyzed the two forms of PPO, with the sole exception of bromelain, where traces of active PPO can still be detected. On these SDS-PAGE gels, we clearly noticed that the hydrolysis of the latent form and the active form by the protease preparations did not happen in the same manner. The latent PPO disappeared before the active PPO even though the band of the latent PPO was initially stronger (cf. the control lane). This is mostly caused by limited proteolysis of latent PPO giving rise to active PPO. Limited proteolysis of PPO has been shown to activate latent PPO (Tolbert, 1973; King & Flurkey, 1987; Robinson & Dry, 1992). Robinson and Dry (1992) reported that latent PPO from broad bean is more sensitive to proteolytic cleavage at the C-terminal end. They found that in the presence of proteases, the latent form of PPO can be cleaved yielding the active form (45 kDa). This suggests that, initially, the proteases attack the exposed peptide chains, thereby liberating or destroying the C-terminal domain, which leads to the generation of active PPO. The active PPO bands, however, tended to disappear as the incubation progressed to 2 h, resulting in a complete breakdown of the two domains of PPO, unveiling the importance of sufficient incubation time for PPO degradation.

3.2. Inhibition with protease preparations

Fig. 2 shows the effect of different concentrations (50, 100, 200, and 500 µg) of protease preparations (papain, calotropain, ficin, and bromelain) on PPO activity. The results indicate that with low concentrations of protease preparations there is an increase in PPO activity during the first minutes of incubation, where 50 µg of papain and calotropain increased PPO activity to 148 ± 3.0% and 106 ± 5.7%, respectively. In addition, ficin and bromelain increased PPO activity to 127 ± 4.0% and 129 ± 1.8% after 2 and 5 min of incubation, respectively. As reported above, this can be attributed to the limited proteolysis of latent PPO, which leads to PPO activation. Observations of the same nature were made by Tolbert (1973), who reported that latent PPO of spinach (*Spinacia oleracea* L.) can be activated in < 30 *sec* by incubation with trypsin.

However, PPO activity started to decrease rapidly as the incubation progressed. The results (Fig. 2A, B, C, D.) show that the inhibition of PPO with proteases was proportional to the time of incubation and the protease concentration. All protease preparations successfully inhibited PPO. However, compared to ficin and bromelain, strong PPO inhibition was clearly demonstrated with the use of papain and calotropain. The papain preparation was the most effective inhibitor for PPO activity, where no PPO activity was detected with all tested papain concentrations after one hour of incubation. These results suggest that papain had a strong proteolytic activity on PPO, which confirms the results of PPO proteolysis monitored by SDS-PAGE. With calotropain, CI was reached after more than 1 h for all protease loads ≤ 100 µg. For ficin, CI was registered with 100, 200 and 500 µg after incubation times of 24, 16

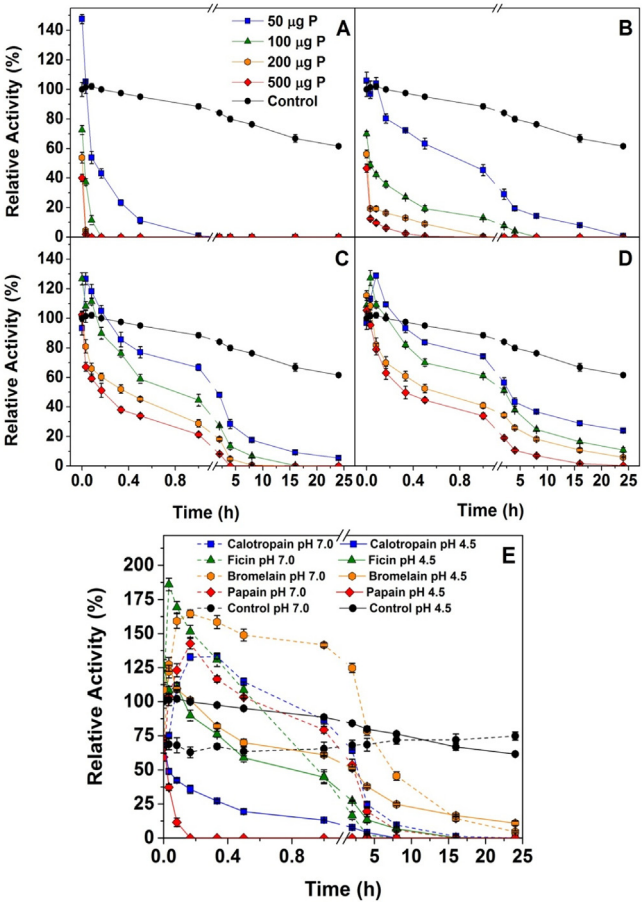

Fig. 2. (A), (B), (C), (D): Effect of the concentration of protease preparations (P) and incubation times on PPO activity at pH 4.5 (A, papain; B, calotropain; C, ficin; D, bromelin). (E) Effect of 100 µg of protease preparations and incubation times on PPO activity at pH 4.5 and 7.0. (//), indicate the gap that separates the scales on the same axis.

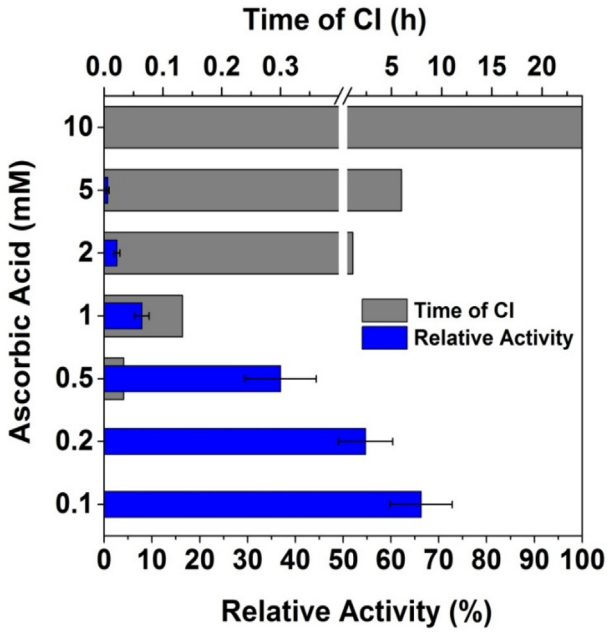

Fig. 3. Effects of different concentration of ascorbic acid on PPO activity and time of complete inhibition (CI). (//), indicate the gap that separates the scales on the same axis.

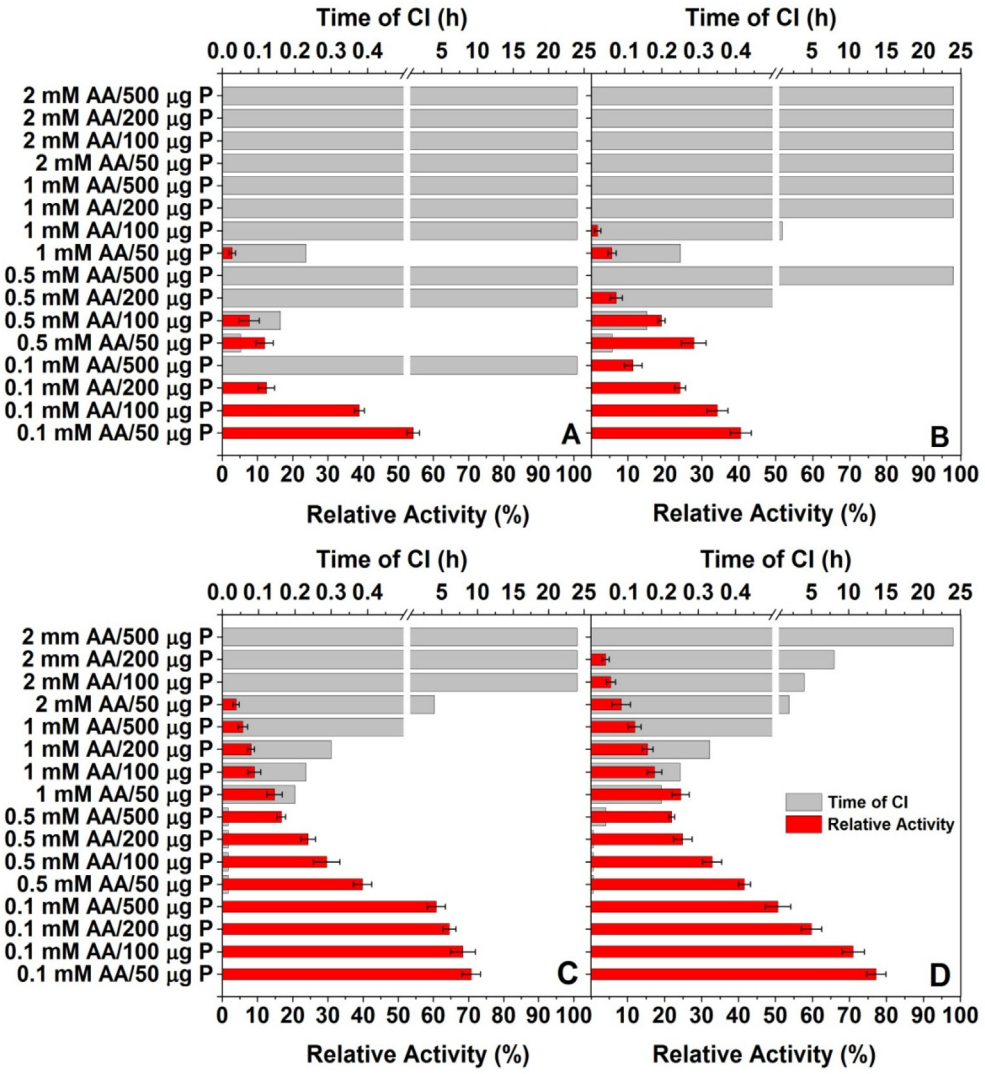

Fig. 4. Effects of different combinations AA/P on PPO activity and time of CI. (A) papain. (B) calotropain. (C) ficin. (D) bromelain. (//), indicate the gap that separates the scales on the same axis.

and 4 h, respectively, while bromelain was the weakest inhibitor, as no CI was registered for all the tested concentrations. According to those results, a complete inactivation of PPO by proteases cannot be achieved without allowing for the appropriate time of incubation, necessary for PPO digestion. The time of incubation can last from a few minutes to several hours, depending on the concentration and the proteolytic activity of the proteases. Furthermore, a short time of incubation may lead to PPO activation and thus an actual increase in PPO activity.

The effect of different protease preparations on PPO activity at pH 7.0 was also studied using 100 µg of each protease preparation. The results (Fig. 2E) show that PPO activation with protease preparations at pH 7.0 during the first minutes of incubation was much higher than for the treatment at pH 4.5 for all four tested proteases. The treatments at pH 7.0 did harm the inhibition process, where the inactivation of PPO was less effective than at pH 4.5. This can be attributed to the difference of the optimum pH between the latent and the active form of PPO, suggesting that the resulting active PPO (after the limited proteolysis) is more stable and active at pH 7.0. Similar observations were reported by Molitor et al. (2015) for PPO from the petals of *Coreopsis grandiflora*, where the latent and the active form of PPO were optimally active at two different pH values of 4.0 and 6.0, respectively. The authors related this difference to the acidic activation of the latent form at pH 4.

3.3. Inhibition with AA

Fig. 3 shows the time of complete inhibition (CI) and the remaining relative activity obtained with AA at concentrations ranging from 0.1 mM to 10 mM. The inhibitory effect of AA on PPO increased markedly with the concentration. At low concentrations (0.1 and 0.2 mM) PPO relative activity decreased significantly to 66 ± 6.5 and 55 ± 5.7%, respectively, and thus, no CI was registered and the browning started immediately. However, for all the concentrations of AA beyond 0.2 mM, we observed a CI of PPO. The time of CI clearly increased with AA concentration. The concentrations 0.5, 1 and 2 mM provided CI for 2, 10 and 70 min, respectively. Increasing the concentration to 5 mM of AA, PPO activity was suppressed for more than 6 h, with only 0.76 ± 0.31% relative activity remaining after this time. Furthermore, the concentration of 10 mM did not allow for PPO activity over the time course of the experiment, yielding more than 24 h of CI. These findings suggest that AA loses its efficacy over time, preventing browning just for a limited time period, which depends on the used concentration. Similar observations were also reported by Gil, Gorny, and Kader (1998), who indicated that AA treatments reduced browning, but only for a finite period of time. AA reduces the oxidized substrate molecules to their diphenolic state, but once it is consumed PPO starts to recover activity.

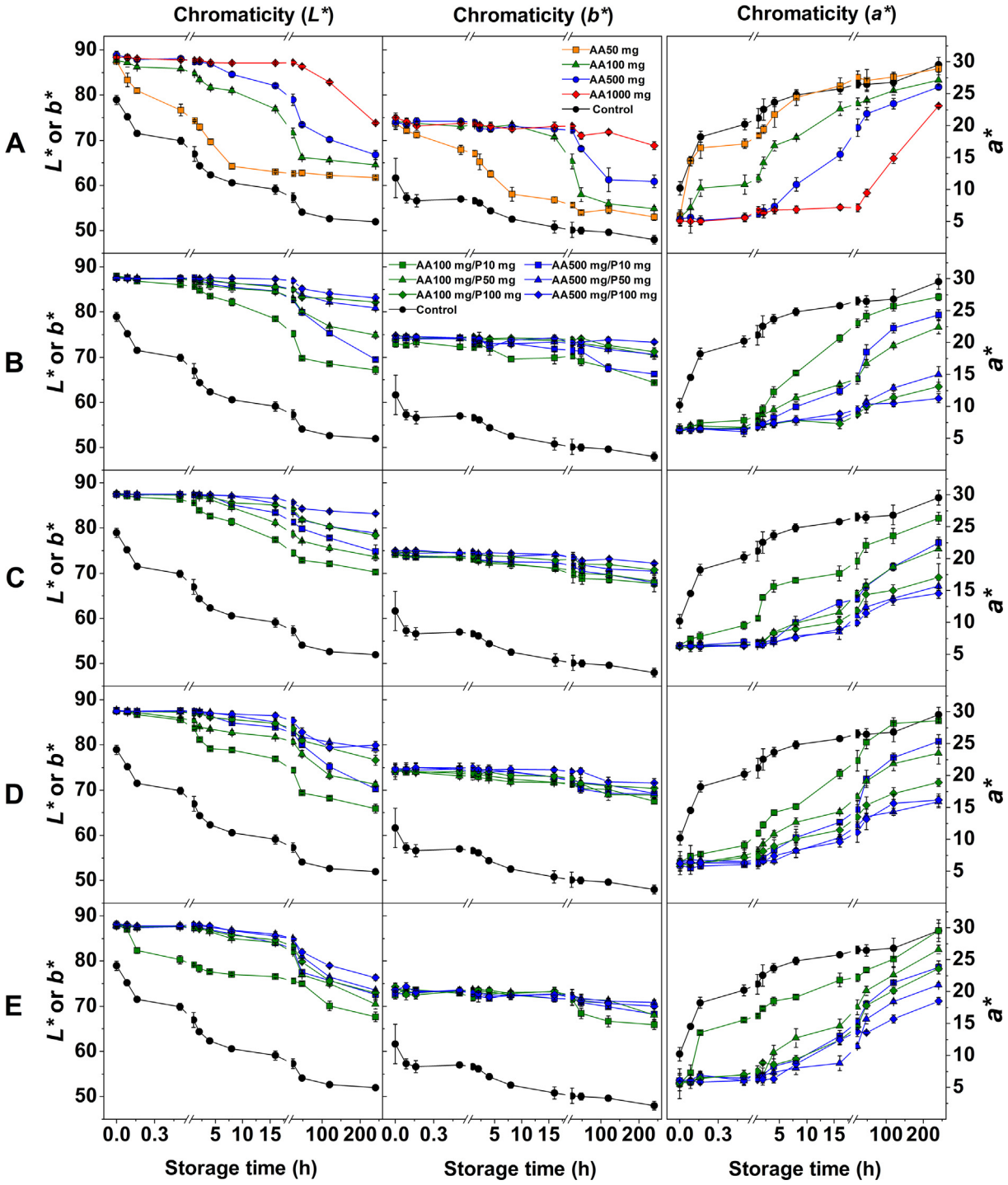

Fig. 5. Chromaticity (a^*, b^*, and L^*) evolution of treated and untreated apricot purees during storage at 4 °C. (A) samples treated with different concentration of AA. (B), (C), (D), (E): samples treated with AA/P combinations (B, AA/papain; C, AA/calotropain; D, AA/ficin; E, AA/bromelain). Each value is presented as mean ± SE (n = 3). (//), indicate the gap that separates the scales on the same axis.

3.4. Inhibition with combinations of AA/P

To optimize the inhibition of PPO by proteases, AA was used to provide inhibition during the time that proteases need to hydrolyze PPO. The concentrations of AA (5 and 10 mM) that gave a CI greater than 5 h in the previous experiment were excluded from the set of tested AA/P combinations. The results are depicted in Fig. 4. The majority of the combinations with AA/papain and AA/calotropain exhibited a CI of PPO activity over 24 h, mostly when papain and calotropain were combined with 1 and 2 mM of AA. However, when papain and calotropain were combined with low concentrations of AA (0.1 and 0.5 mM), PPO activity was detected, especially for the combinations with 50 and 100 μg of protease preparations. This indicates that AA was oxidized before the proteases could complete the hydrolysis of PPO. AA/ficin and AA/bromelain combinations were not as effective as AA/papain and AA/calotropain. This can be justified by the low proteolytic activity of ficin and bromelain compared to papain and calotropain (see Fig. 2), which requires a higher concentration of AA to allow for the time needed for a sufficiently complete proteolysis of PPO. However, even if those combinations did not give a CI over 24 h, the overall

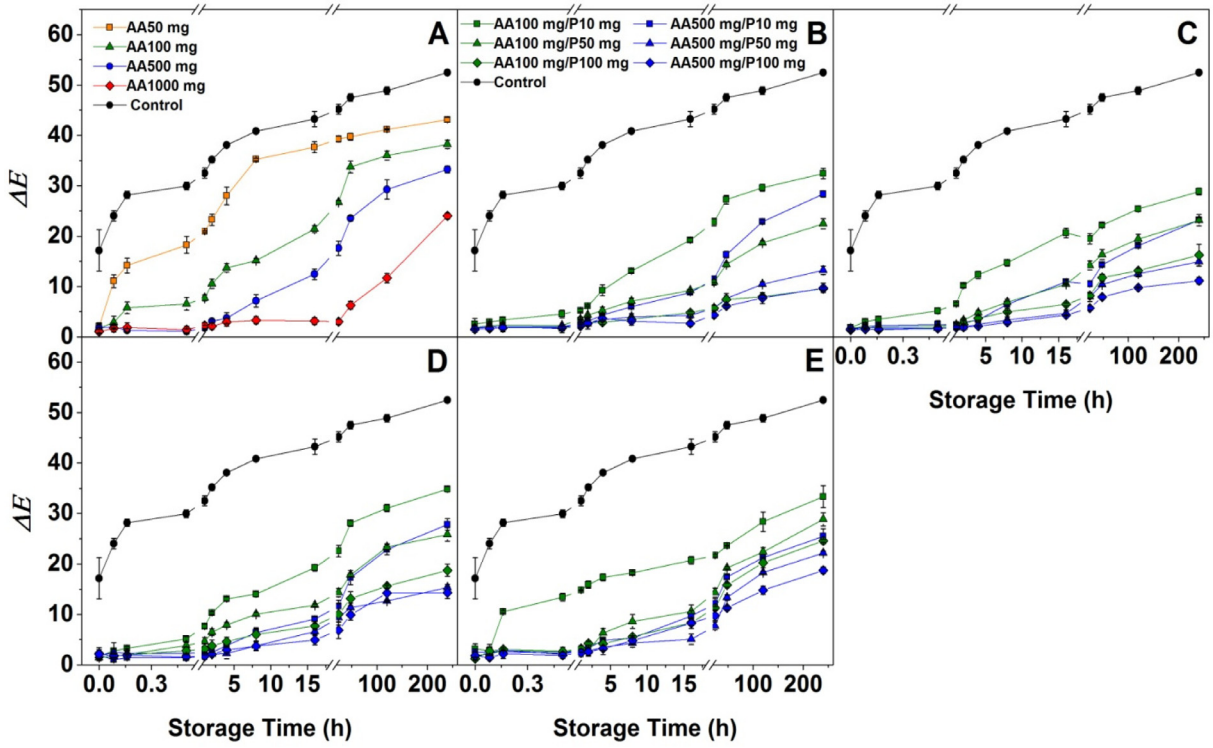

Fig.6. Change in total color difference (ΔE) of treated apricot purees during storage at 4 °C. (A) Samples treated with different concentrations of AA. (B), (C), (D), (E): samples treated with different AA/P combinations (B, AA/papain; C, AA/calotropain; D, AA/ficin; E, AA/bromelain). Each value is presented as mean ± SE (n = 3). (//), indicate the gap that separates the scales on the same axis.

results show that they did markedly improve the time of CI compared to the treatments with AA only. We suggest that the proteolysis of PPO by proteases decreased the PPO activity, thereby, also decreasing the amount of the produced quinones, which directly increased the time of CI by lowering the amount of consumed AA.

3.5. Application on apricot puree

In order to test the efficiency of the AA/P combinations *in vivo*, tests with samples of 100 g fresh apricot puree were conducted. The changes in color coordinates (a^*, b^*, and L^*) and the changes of total color difference (ΔE) were measured over 10 days and the results are presented in Figs. 5 and 6, respectively. In order to assess the efficiency of treatments, ANOVA was performed on the color parameters at the end of the storage period (10 days, Supplementary Table 1).

Generally, the browning of fruits and vegetables is marked by a decrease of L^* values and an increase of a^* values, which consequently increases ΔE values. The overall appearance of apricot purees regardless of treatment was a gradual decrease in a^* and L^* values and a gradual increase in b^* and ΔE values as storage time progressed. However, important differences were found between the different samples. In recently homogenized untreated puree (control), a^*, b^*, and L^* coordinates were 10 ± 1.1, 62 ± 4.3 and 79 ± 1.0, respectively. These values are significantly different from all the recently homogenized, treated samples (Fig. 5). This difference indicates high browning in the control puree, reflecting an immediate browning that mainly occurred during the homogenization. Additionally, the browning of the control puree increased gradually during storage time, and ΔE moved from 17 ± 4.1 at 0 min to 52.5 ± 0.23 at day 10, where 50% of this browning occurred within the first 10 min. Treatments with 100, 500 and 1000 mg of AA were effective in delaying the browning of apricot puree for 5 min, 30 min and 24 h, respectively, compared to the untreated samples (Fig. 6A). However, browning manifested itself afterwards, indicated by clear declines of chromaticity L^* and b^* and an increase of chromaticity a^*, as well as ΔE values.

In comparison to untreated apricot purees, all AA/P combinations significantly ($p < 0.0001$) decreased browning and the more AA and/or protease was applied, the longer the apricot puree retained its color (Supplementary Table 1). The results clearly show that the AA/P combinations were more effective than the application of AA alone, and they reduced browning considerably, for some combination browning was reduced by 80% (500 mg AA/100 mg papain, 100 mg AA/100 mg papain, and 500 mg AA/100 mg calotropain). The apricot purees treated with AA/papain combinations had the lowest browning during storage time, where AA/calotropain was slightly less efficient followed by the AA/ficin and AA/bromelain combinations. These observations are in accordance with our findings *in vitro*. Meanwhile, no combination did manage to completely stop browning reactions *in vivo* like they did *in vitro*. A complete control of enzymatic browning *in vivo* was always a difficult goal to achieve even with strong inhibitors (McEvily et al., 1992; Marshall et al., 2000) or blanching (Deylami, Rahman, Tan, Bakar, & Olusegun, 2016), where treated fruits and vegetables mostly still undergo minimal browning. However, even if the main cause of color changes of apricot puree is attributed to PPO, the browning *in vivo* can be partially caused by other phenomenon, who may also have contributed to the color change observed here. The liquid release caused by the mechanical stresses favors fluids leakage, thereby causing a decrease in the water holding capacity of the puree over the time of storage, which leads to lower reflectance and a decrease in chromaticities $L*$ and $b*$ (Zhang et al., 2018). Different reactions may also simultaneously contribute to color changes of the puree such as non-enzymatic browning and the oxidation of carotenoids and AA (De Ancos, Sánchez-Moreno, Plaza, & Cano, 2011).

4. Conclusion

The results presented herein indicate that the selected plant protease preparations were able to efficiently inactivate apricot PPO. The

degree on inactivation is proportional to the time of incubation, the proteolytic activity and the concentration of the protease and thus, papain and calotropain were the most effective proteases on PPO. The AA/P combinations were highly effective as browning inhibitors, where ascorbic acid prevented the browning reactions that may occur before or during the proteolytic inactivation of PPO with the proteases. In addition, the application of AA/P combinations on apricot puree significantly reduced color loss of the treated samples, thereby demonstrating the feasibility of the concept in a real-world setup. This study provides clear proof that papain, calotropain, ficin, and bromelain can be effective as natural inhibitors if there is enough time to digest the PPO in question. Therefore, proteases in combination with ascorbic acid can be used as substitutes for chemical inhibitors of enzymatic browning.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2019.100053.

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