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Characterization of polyphenols compounds extracted from stressed apple peel and their interaction with β-lactoglobulin

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ABSTRACT

This paper proposes to apply a postharvest environmental stress to red apples, *Malus domestica*, variety Red Delicious in order to increase the polyphenols compounds (PP) content in their peels. The possibility of enhancing extractable PP provides a useful alternative for the use of discarded crops in the food industry. A great increase in PP was observed in response to light damage produced by the environmental stress applied in this work. Flavonols > anthocyanins > flavanols > dihydrochalcones > phenolic acids is the order in PP content. The interaction of the extracted PP from unstressed and stressed apple peels with beta-lactoglobulin (β-LG) was characterized. A PP/β-LG complex which was formed with one single binding site in the protein was determined. The interaction was spontaneous and enthalpy driven. PP extracted from unstressed samples had greater affinity for the protein than PP extracted from stressed samples, possibly due to the polar characteristic of anthocyanins. The results of this last study could provide a better understanding of the interaction between PP and β-LG to incorporate them into functional foods.

1. Introduction

Polyphenols compounds (PP) exert a protective action on human health promoting beneficial effects on chronic noncommunicable diseases due to their antioxidant properties. Many epidemiological studies correlate PP ingestion with a lower incidence of chronic diseases, such as cardiovascular disease, diabetes and cancer [1]. Due to the evidence of their high antioxidant activity, PP are valued as natural products to be incorporated into functional foods [2].

PP content of plants increases to protect tissues in response to various agents of biotic (living organisms) and abiotic stress (ultraviolet radiation, heat and cold, etc.). This activity is related to the fact that PP have one or more aromatic rings and one or more hydroxyl groups in their molecular structure which react with free radicals forming stable products [3].

Supercritical fluid extraction is already a well-established method to obtain PP in industrial scales. However, other methods such as ultrasound-assisted extraction, microwave-assisted extraction, and pressurized-liquid extraction are also gaining ground in the food

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industry [4].

Apple fruit is a major source of PP, with the highest content located in their peel [5]. Apple is the most relevant crop in the Alto Valle de Río Negro (Patagonia, Argentina) where 85% of the production is located [6]. 50% of the harvest is industrialized (apple juices, jam, etc.), 22% is exported to other countries and 28% is distributed to fresh consumption. 8% of the total production is discarded because of the low demand or they do not fulfil quality requirements [7]. The dominant commercial apple variety in Argentina is Red Delicious.

The high content of PP in apples has a commercial interest to producers and food industry as they can find alternative uses for discarded fruits. With this in mind, it is novel to study the increase of PP in apple peel by applying controlled abiotic environmental stress to whole apples, aiming to use them as a source of PP in the food industry.

On the other hand, instability and poor bioavailability of hydrophobic and amphiphilic compounds limit its application in functional foods, but the formation of complexes improves their release, stability and bioavailability [8]. In this sense, protein binding process presents a great potential for the development of innovative products in the food industry. The major whey protein, beta-lactoglobulin (β-LG), has been extensively studied in its ability to bind hydrophobic and amphiphilic compounds such as flavorings, vitamins, fatty acids and PP [9–11]. β-LG is a compact globular protein (18.3f kDa) with a three-dimensional structure consisting of eight strands of antiparallel β-sheet twisted into a cone-shaped barrel (hydrophobic pocket) and a short α-helix strand [12].

Considering the potential utilization of discarded apples as a source of polyphenols (PP), the objective was to research the increase of PP content in apple peels through the application of controlled postharvest abiotic environmental stress. Furthermore, the interaction between PP extracted from apple peels and the milk protein β-lactoglobulin (β-LG) was assessed. The findings of this study have the potential to enhance comprehension of the PP-β-LG interaction in functional foods and offer alternative avenues for repurposing discarded apples from the industry.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteau reagent, gallic acid (GA), 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramxethylchroman-2-carboxylic acid (Trolox) and acrylamide were purchased from Sigma-Aldrich Chemical Co. (St-Louis, MO, USA). β-LG was supplied by DAVISCO Foods International, Inc. (Le Sueur, MN). All other chemicals were of analytical grade.

2.2. PP analysis from apple peels

2.2.1. Plant material and sample preparation

A total of 80 apples of Red Delicious variety (were collected from the same tree) during the commercial harvest on February 15th, 2021, at the Alto Valle de Río Negro (Argentina) and then transported to the laboratory in a refrigerated truck. Once the apples arrived at the laboratory (one day after being harvested), they were washed, drained and dried with paper. After cleaning, the apples were immediately used for further studies [13].

2.2.2. Environmental stress

Twelve apples were submitted to environmental stress. The environmental stress consisted of apples exposure to natural air and light, protected from strong wind as well as rain. The average daily temperature was 25 °C and the average daylight was 15 h. Three apples were withdrawn at 0 (unstressed sample: US) and every 7 days (stressed samples SSX, being X the days of stress). Immediately, the apple peel was separated and the corresponding PP extraction was performed.

2.2.3. PP extraction

PP were extracted from the apple peels using a method adapted from Ref. [5]. 5 g of apple peel (US and SSX) was separated by using a scalpel blade, cut into small pieces and then mixed with 20 mL of 80:20 cold methanol/water solution for 5 min. The sample was then homogenized for 3 min using a D-500 Homogenizer (Dragon Lab, Beiging, China). The suspension was filtered through Whatman No. 1 filter paper in a Buchner funnel under vacuum. The retained solids were put into 15 mL of 80% methanol and the mix was homogenized for 3 min before being re-filtered. All the filtrates obtained were collected and evaporated to dryness using a rotary evaporator at 45 °C (Buchi Labortechnik AG CH-9230, Flawil, Switzerland). The dried extracts were stored at – 18 °C protected from light and atmospheric oxygen. The extracts were reconstituted with 3.5 mL 20 mM phosphate buffer, pH 6.8 and used immediately. All extracts were done in triplicate. The extracts were used for the determination of total phenolic content (TPC), antioxidant capacity (AC), and PP concentrations using reverse phase high performance liquid chromatography (RP-HPLC).

2.2.4. TPC determination

TPC was determined by the Folin-Ciocalteau method [14]. Aliquots of US and SSX samples were mixed with 2 mL of Folin–Ciocalteu reagent previously diluted 1:10 with distilled water and 1 mL of 7.5% sodium carbonate solution. The mixture was incubated in a water bath at 40 °C for 10 min. The absorbance was read at 765 nm on a UV–Vis spectrophotometer (Jasco V-550, Tokyo, Japan). Measurements were compared to a standard curve of GA prepared in a concentration range of 0–500 μg mL^{-1}. The results were expressed as mg of gallic acid equivalents GA per 100 g of fresh weight peel (FW) (mg GAE/100 g FW). All the samples

were analyzed in triplicate.

2.2.5. AC determination

In order to estimate AC, ABTS radical cation decoloration ($ABTS^{+}$) assay was used [15]. The determination was performed by mixing aliquots of each PP sample assayed with $ABTS^{+}$ (dissolved in methanol) and measuring the absorbance at 734 nm at 6 min after mixing. Results were obtained by interpolating the absorbance on a calibration curve constructed using Trolox as standard (range between 0 and 2.5 mmol L^{-1}). These results were expressed in μmol Trolox equivalents per g of FW peel (μmol TEAC/g FW). All the samples were analyzed in triplicate.

2.2.6. RP-HPLC analysis

The levels of PP on apple peel were analyzed by RP-HPLC. The chromatographic analysis was performed on US and SSX with the highest TPC value. For this purpose, an Agilent HPLC 1260 Infinity II Quaternary System (Agilent Technology, Santa Clara, CA, USA) was used. The system was equipped with a photodiode array detector (PDA) and a vialsampler. A Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm) and a Poroshell 120 EC- C18 guard column were employed for the separation of PP compounds. RP-HPLC settings and PP analysis were in accordance with a methodology previously reported [16–18]. PP present in the different samples were identified by comparison retention times and spectrum peaks (200–600 nm) with authentic PP standards. Chlorogenic acid (Cha), (+)-catechin (Ca), (−)-epicatechin (ECa), quercetin-3-*O*-glucoside (QGlu) and quercetin (Q) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Procyanidin B1 (PB1), procyanidin B2 (PB2), cyanidin-3-*O*-galactoside chloride (CGa), quercetin-3-*O*-galactoside (QGa), quercetin-3-*O*-rhamnoside (QRh) and phloretin-2′-*O*-glucoside (PhGlu) were obtained from Extrasynthese (Genay, France). The identified PP were quantified by using calibration curves of authentic standards. For the tentative identification of quercetin-3-*O*-xyloside (QXy), a quercetin calibration curve was used. The three extracts of US and the selected SSX samples were injected to obtain PP concentration. All samples were analyzed in duplicate.

The relative change (RC) in the content of each PP subclass between SSX and US was calculated using equation (1):

$$(SSX)_{PPS} / (US)_{PPS} \quad (1)$$

where $(SSX)_{PPS}$ and $(US)_{PPS}$ correspond to the content (in percentage) of each PP subclass for US and SSX samples, respectively.

2.3. Interaction between β-LG and PP

The methodology employed in this work to study PP/β-LG binding was based on quenching measurements of intrinsic protein fluorescence promoted by PP [18]. For this purpose, fluorescence spectra were recorded on a Jasco FP-770 spectrofluorometer (Jasco International Co, Tokyo, Japan). Aliquots of 3 mL of 20 μM of protein (20 mM phosphate buffer, pH 6.8) were placed in the cuvette of the spectrofluorometer and titrated with stock solutions of US and SSX samples. After the addition of each aliquot fluorescent,

Table 1
Phenolic content and percent for US and SS14.

Polyphenol subclass	US		SS14		Increase %
	(mg kg^{-1} FW)	%	(mg kg^{-1} FW)	%	
Cha	11.58 ± 0.38[a]		35.80 ± 0.95[b]		209
Total phenolic acids	11.58 ± 0.38[a]	1.4	35.80 ± 0.95 [b]	1.6	209
PB1	28.90 ± 0.45[a]		58.95 ± 0.85[b]		104
Ca	58.43 ± 3.85[a]		116.78 ± 4.65[b]		100
PB2	55.58 ± 1.88[a]		134.48 ± 4.45[b]		142
ECa	42.25 ± 1.20[a]		111.70 ± 2.58[b]		164
Total flavanols	185.15 ± 7.38[a]	22.9	421.90 ± 12.53 [b]	18.4	128
CGa	166.25 ± 9.30[a]		668.23 ± 19.05[b]		302
Total anthocyanins	**166.25 ± 9.30[a]**	**20.5**	**668.23 ± 19.05[b]**	**29.1**	**302**
QGa	115.00 ± 3.68[a]		278.73 ± 7.65[b]		142
QGlu	76.98 ± 4.70[a]		210.18 ± 10.35[b]		173
Q	0.78 ± 0.03[a]		1.05 ± 0.05[b]		35
QXy	42.43 ± 2.25[a]		124.15 ± 3.93[b]		193
QRh	58.75 ± 2.55[a]		181.30 ± 7.68[b]		209
Total flavonols	**293.93 ± 13.20[a]**	**36.3**	**795.40 ± 29.65[b]**	**34.7**	**171**
PhGlu	153.00 ± 4.35[a]		371.48 ± 6.53[b]		143
Total dihydrochalcones	**153.00 ± 4.35[a]**	**18.9**	**371.48 ± 6.53[b]**	**16.2**	**143**
Total Polyphenol	**809.9 ± 34.62[a]**	**100**	**2292.8 ± 68.72[b]**	**100**	

Results were expressed as x ± DS. Different letters in a row mean significant differences ($p < 0.05$) among samples. 3rd and 5th columns correspond to the content (in percentage) of each PP subclass for US and SS14, respectively. 6th columns correspond to the percentage increase in polyphenol content.

Abbreviations: Cha, chlorogenic acid; PB1, procyanidin B1; Ca, (+)-catechin; PB2, procyanidin B2; ECa, (−)-epicatechin; CGa, cyanidin-3-*O*-galactoside chloride; QGa, quercetin-3-*O*-galactoside; QGlu, quercetin-3-*O*-glucoside, Q, quercetin; QXy, quercetin-3-*O*-xyloside; QRh, quercetin-3-*O*-rhamnoside and PhGlu, phloretin-2′-*O*-glucoside.

spectrum was immediately recorded (λ excitation 280 nm and λ emission 250–450 nm) and the maximum fluorescence intensity was determined. All titration curves were performed in triplicate.

Molar concentration of PP solutions was an apparent concentration ($[PP]_{app}$) since the samples were heterogeneous. In that sense, $[PP]_{app}$ was calculated using an average number of molecular weights (M_n) determined from the data obtained from RP-HPLC analysis (Table 1) [19]. M_n was calculated using equation (2):

$$M_n = \sum w_i \Big/ \sum n_i \tag{2}$$

where w_i is the weight concentration in g of individual PP and n_i is the corresponding number of moles of individual PP per L. The molecular weight of individual PP used for the calculation of M_n was: Cha - 354, Ca - 290, ECa - 290, QGlu - 463, Q - 302, PB1 - 579, PB2 - 579, CGa - 449, QGa - 434, QRh - 448, PhGlu - 449 and QXy – 434 g mol^{-1}. Fluorescence quenching is described by the Stern-Volmer equation (3):

$$F_0 \Big/ F = 1 + K_{SV}\ x\ [PP]_{app} \tag{3}$$

where F_0 and F are the maximum fluorescence intensities before and after addition of PP; K_{SV} is the Stern-Volmer quenching constant and $[PP]_{app}$ is the apparent molar concentration of the quencher. The measured fluorescence intensity was corrected due to the inner filtering effect according to Shpigelman et al. (2010) [20].

Fluorescence quenching between ligands and proteins may be a result of a static or dynamic mechanism. A static quenching implies a complex formation between fluorophore and quencher, whose stability decreases when temperature increases. To obtain static quenching parameters (binding sites number, n and association binding constant, K_a), a double double-logarithmic equation (4) was used [21]:

$$\log[(F_0 - F) / F] = \log K_a + n \log[PP]_{app} \tag{4}$$

The dominant forces for the PP/β-LG interaction can be determined by the values of enthalpy change (ΔH°) and entropy change (ΔS°), which can be calculated by equations (5)–(7):

$$K_a(T_2) / K_a\ (T_1) = (\Delta H^\circ / R)\ [1 / T_1 - 1 / T_2] \tag{5}$$

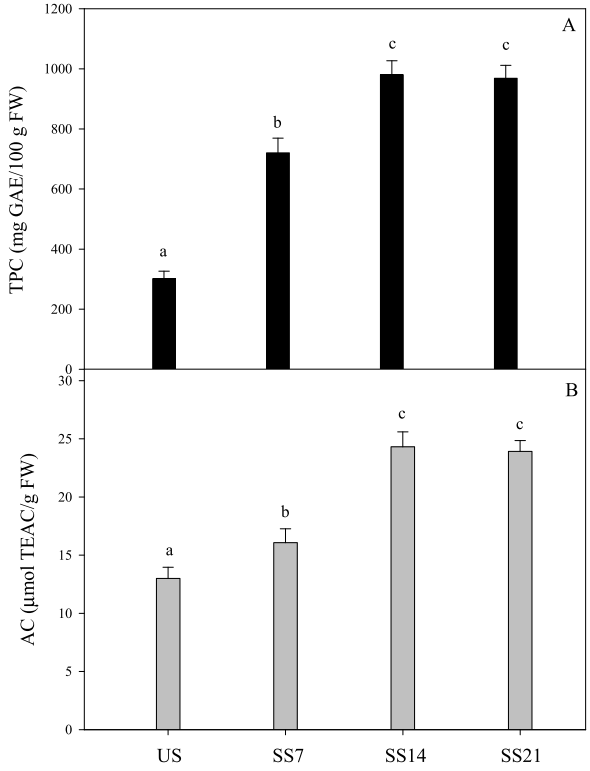

Fig. 1. (A) Total Phenolic Content (TPC) vs. days of environmental stress. Each value is the mean of three replicates and error bars indicate standard deviations. Different letters above columns indicate significant differences ($p < 0.05$). (B) Antioxidant Capacity (AC) vs. days of environmental stress. Each value is the mean of three replicates and error bars indicate standard deviations. Different letters above columns indicate significant differences ($p < 0.05$).

$$\Delta G^{\circ} = -RT\ln K_a(T) \quad (6)$$

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} \quad (7)$$

where ΔG° is the free energy change, T is the experimental temperature and R is the gas constant (8.314 J mol^{-1} K^{-1}).

All the fluorescence studies were performed at 298 K (T_1) and at 308 K (T_2).

2.4. Statistical analysis

All data were reported as mean ± standard deviation of replicates and analyzed using Statgraphics plus 3.0 software. Differences between the means were established using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences at $p < 0.05$ were considered significant.

3. Results and discussion

3.1. TPC and AC of US and SSX

The value of TPC determined in the apple peel of US samples was 301.3 ± 25.0 (mg GAE/100 g FW) ($p < 0.05$) and the corresponding AC value was 13.0 ± 0.5 (μmol TEAC/g FW) ($p < 0.05$). These values were closed to those published by several authors for red apples picked at maturity [5,18,22,23,24]. A significant increase in TPC and AC was observed in stressed samples during exposure to environmental stress, Fig. 1A and B, respectively. The highest TPC value was reached at 14 days of stress, which represented an increase of 225% over US. Following the same trend, the highest AC value was also reached at 14 days of stress (87% of increase). It can be concluded that the environmental stress applied was an effective treatment for increasing PP content and antioxidant properties. It should be noted that two considerations must be taken into account regarding TPC and AC determinations. First, Folin-Ciocalteau reagent can overestimate PP content because it also reacts with any reducing substances present in the sample (sugars, amino acids, vitamins, etc.) and not only with phenolic compounds [25,26]. Second, different PP do not show the same antioxidant activity, being CGa the polyphenol subclass that presents the highest concentration in apples [27].

TPC and AC at 21 days showed no significant differences compared to the values obtained at 14 days of environmental stress. In addition, apple decomposition began to be evident after 21 days of stress. In view of these results, SS14 was selected for further studies.

3.2. PP analysis on US and SS14 by RP-HPLC

Table 1 shows the content of PP subclasses and individual PP identified in the peel of Red Delicious harvested in the Alto Valle de Río Negro (Argentina). These compounds belong to five different subclasses. The content pattern of PP subclasses determined in US samples followed the order: flavonols > flavanols > anthocyanins ~ dihydrochalcones > phenolic acids. This result was consistent with those reported by Ref. [17] for the *Cortland* variety which is among the most popular red apples consumed in the United States and Canada. The two apple varieties presented a flavonols/flavanols ratio of 1.6. For both varieties, the more concentrated flavonols were QGa and QGlu, and the more concentrated flavanols were Ca and PB2. The content in anthocyanins was the major difference between them, since for Red Delicious 20.5% of total PP was CGa (Table 1) while for *Cortland* variety was only 5.0% [18].

It can be seen that all the PP identified in the present study increased their amount in apple peel due to environmental stress (Table 1). The content of each PP subclass for SS14 samples followed the order: flavonols > anthocyanins > flavanols > dihydrochalcones > phenolic acids. The most important environmental stress factor to which apples were subjected in this study was

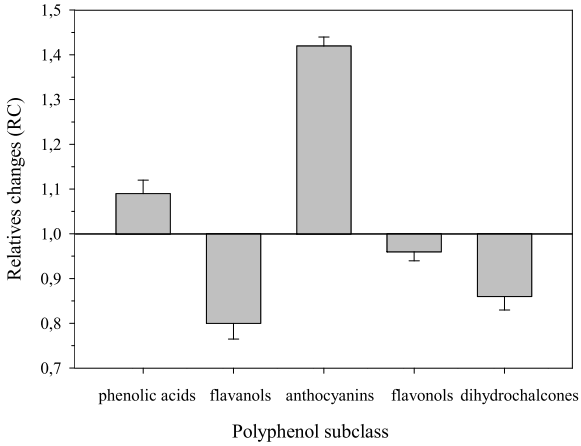

Fig. 2. Relative change in the percentage content of each PP subclass between SS14 and US (SS14/US).

exposure to light, since temperature moderately changed during the assays. Thus, the PP increment observed was a response to the damage caused by light. Sharma et al. (2019) [3] reported that flavonols and anthocyanins act as light screens due to their capability of absorbing both visible and UV radiations, hence protecting plants from this harmful factor.

On the other hand, RC was calculated in accord with Equation (1) for each PP subclass taking into account the percentages shown in Table 1 (Fig. 2). Two PP subclasses had RC values higher than one while the other three subclasses had lower values. These data indicate that anthocyanins increased their RC by 42.0% and phenolic acids by 14.3%, while flavanols decreased their RC by 19.6%, dihydrochalcones by 14.3% and flavonols by 4.4%. These last results indicated that anthocyanins showed the major increment of RC between the different PP assayed in this work (anthocyanins > phenolic acids > flavonols > dihydrochalcones > flavanols).

3.3. Interaction between β-LG and PP

Table 1 shows the most representative monomeric PP present in red apple peel studied in this work. Besides this, polymeric PP such as procyanidins, cannot be determined by RP-HPLC [17]. Thus, these two factors determined that total PP concentration was underestimated by $[PP]_{app}$ values.

On the other hand, for both US and SS14, K_{SV} values decreased with increasing temperature from 298 K to 308 K, indicating that protein fluorescence quenching was due to a static complex formation between PP and β-LG [20] (Fig. 3A and Table 2). This behavior was also observed by other authors for the interaction between this milk protein and pure PP such as Cha [28] and cyanidin-3-*O*--glucoside [29], with K_{SV} values similar to those determined in this study.

In addition, Table 2 shows the binding constants (data taken from Fig. 3B) and thermodynamic parameters for the interaction between β-LG and US and SS14 at the two temperatures assayed. K_a values obtained from Equation (4) decreased with increasing temperature for both samples as it occurred with K_{SV} values, confirming a static quenching process. The binding sites number was found to be approximately 1, indicating that there was only one single binding site for PP inside the protein as was reported by several authors [28–30]. It has been to be noted that K_a diminished for SS14 with respect to US samples. This fact was related to the great increase in the number of moles of CGa in SS14 samples (from 20.5% in US to 29.1% in SS14). Anthocyanins, the only PP present in these red apple peels, have a net positive charge in its structure. This polar characteristic could be responsible for the observed decrease of K_a between US and SS14 samples [31]. ΔG° had a negative value, indicating that the binding process was spontaneous (Table 2).

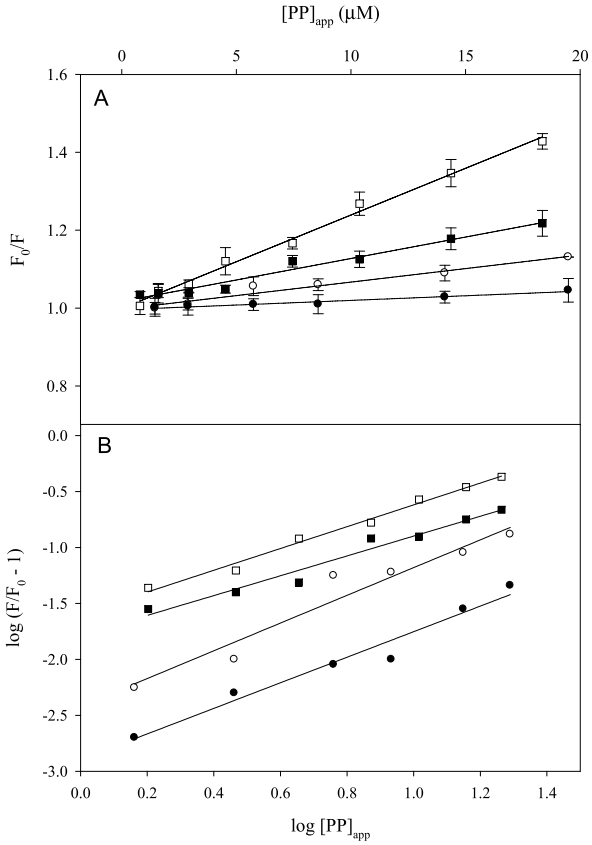

Fig. 3. (A) Stern-Volmer plots for the quenching of intrinsic fluorescence of β-LG by US and SS14 at two temperatures. (B) Double logarithmic plots for the interaction between US and SS14 with β-LG at two temperatures. (□) US at 298 K and (■) US at 308 K. (○) SS14 at 298 K and (●) SS14 at 308 K.

Table 2
Quenching binding constants and thermodynamic parameters for PP-β-LG interaction.

Compound	T (K)	K_{SV} (10^{-4} M^{-1})	K_a (10^{-4} M^{-1})	n	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (kJ mol^{-1} K^{-1})
β-LG + US	298	2.40	6.62	0.974	−27.50	−31.17	−0.012
β-LG + US	308	1.10	4.40	0.798	−27.38		−0.012
β-LG + SS14	298	0.69	1.13	1.338	−23.12	−80.21	−0.191
β-LG + SS14	308	0.24	0.39	1.142	−21.21		−0.191

Ross and Subramanian (1981) [31] also described that van der Waals forces and hydrogen bonding might play a major role in the binding process when ΔH° and ΔS° are both negative. Moreover, it can be concluded from the data presented in Table 2 that the binding between PP and β-LG was an enthalpy driven reaction, suggesting that more hydrogen bonds were formed than were broken. This analysis can be applied to both US and SS14 binding to β-LG.

4. Conclusions

PP are valued as natural products to be incorporated into functional foods due to their health benefit properties. In this work it was demonstrated that controlled postharvest environmental stress can be used to increase PP levels in red apple peels. The possibility of enhancing extractable PP yields, provides a useful alternative for the use of discarded crops in the food industry. A great increase in PP was observed in response to the light stress applied in this work. Flavonols > anthocyanins > flavanols > dihydrochalcones > phenolic acids is the order in PP content.

On the other hand, the present work demonstrated the presence of one single binding site for PP inside the protein and that the interaction is an enthalpy driven reaction.

This research constitutes a pioneering effort, as it delves into the effects of stress on red apple peels, simulating a situation akin to discarded apples. It comprehensively examines the composition and quantity of PP and their interaction with β-LG. Concurrently, alternative techniques for PP extraction continue to be investigated. Also, further studies must be done in order to clarify the interaction between PP of apple peels and β-LG. In this context, molecular modeling and docking studies should be employed, given the diverse structures of PP involved in the binding process.

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Author contribution statement

Emilce E. Llopart: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Roxana A. Verdini: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

Néstor J. Delorenzi, Pablo A. Busti: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

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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