Food Bioprocess Technol (2014) 7:1518–1525
DOI 10.1007/s11947-013-1208-3

COMMUNICATION

Effect of *Stevia rebaudiana* on Oxidative Enzyme Activity and Its Correlation with Antioxidant Capacity and Bioactive Compounds

Maria N. Criado · Francisco J. Barba · Ana Frígola · Dolores Rodrigo

Received: 18 April 2013 / Accepted: 29 September 2013 / Published online: 12 October 2013

Abstract The effect of different concentrations of *Stevia rebaudiana* Bertoni (*Stevia*) on polyphenoloxidase and peroxidase activities, antioxidant capacity and other bioactive compounds of a mixture of fruits, during 10 days of incubation at 10 and 37 °C, was studied. A significant decrease ($p<0.05$) in both enzyme activities was observed when *Stevia* was added, showing an inhibitory effect. Samples with a high *Stevia* concentration showed the highest total phenolic content and antioxidant capacity, whereas non-significant changes were found in the ascorbic acid and carotenoid contents of the samples with *Stevia* added. A negative significant dependence ($p<0.05$) during incubation was obtained between enzyme activity and antioxidant capacity, so that antioxidant capacity increases as enzyme activity decreases. Therefore, enzyme activity could be used as an indirect index of antioxidant capacity. The results clearly showed that *Stevia* had significant potential for use not only as a sweetener but also as a natural preservative agent.

Keywords
Stevia rebaudiana · Peroxidase · Polyphenoloxidase · Bioactive compounds · Antioxidant capacity

Introduction

The food industry has shown increased interest in extracts from *Stevia rebaudiana* Bertoni (*Stevia*), because it can be a nutritional strategy to replace or substitute sugar content owing to its high content of sweeteners. Currently, *Stevia* in leaf or extracted forms has been approved as a dietary supplement by the FDA in the USA and under similar classifications in several other countries. In March 2010, after EFSA issued a positive opinion on the safety of steviol glycosides and raised their acceptable daily intake (ADI), expressed as steviol equivalents, to 4 mg/kg body weight/day, the introduction of steviol glycosides on the European market as food additives with a purity of more than 95 % is imminent. Furthermore, in November 2011, the European Commission approved steviol glycosides as food additives (Commission Regulation 2011), which will probably lead to wide-scale use in Europe.

So far, little data has been available regarding the practical applications in foods and stability under different processing and storage conditions (El Nehir and Simsek 2012). It has been reported that *Stevia* is nutrient rich and contains substantial amounts of minerals, vitamins, polyphenols, and other antioxidant compounds that confer antioxidant activity to *Stevia* extracts (Lemus-Mondaca et al. 2012). The methods employed to determine the total antioxidant capacity of these extracts are mainly based on chemical reactions. The ferric-reducing ability of plasma (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays was selected by Tadhani et al. (2007) to find, for the first time, that *Stevia* leaves had strong antioxidant activity; DPPH, hydroxyl radical scavenging activity and superoxide anion radical scavenging activity have been used by Kim et al. (2011), and DPPH and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) tests were chosen by other authors (Muanda et al. 2011). Phenolic compounds have been shown to be good contributors to the total antioxidant capacity of foods (Zulueta et al. 2009). On the other hand, they are substrates of oxidative reactions catalysed mainly by polyphenoloxidase (PPO, EC 1.14.18.1) and, to a lesser extent, owing to the generation of hydrogen peroxide during this oxidation of phenolic compounds, by peroxidase

M. N. Criado · D. Rodrigo (✉)
Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Avda. Agustín Escardino, 7, 46980 Paterna, Spain
e-mail: lolesra@iata.csic.es

F. J. Barba · A. Frígola
Area of Nutrition and Food Chemistry, Universitat de València, Avda. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

(POD, EC 1.11.1.7) (Tomás-Barberán and Espín 2001). However, in the presence of an antioxidant substance, such as ascorbic acid, or any other anti-browning agent (4-hexylresorcinol, glutathione, among others), PPO and POD activity might be depressed. Food safety directs research in a constant quest for inhibitors from natural sources as alternatives to chemical additives, because they are largely free of harmful side effects. Consequently, there is great demand from the food industry for safe, effective PPO inhibitors of natural origin. Recently, several studies have shown that natural agents have an inhibitory effect on PPO, including honey (Chen et al. 2000) and some aerial plant parts (Loizzo et al. 2012). In this respect, in view of the composition of *Stevia*, it is interesting to study the capacity of extracts of this plant as inhibitors of enzymatic browning caused by POD and PPO and their potential use in the food industry as natural preservatives and as a sweetening additive. Moreover, the inhibition of enzyme activity leads to a reduction in the degradation of phenolic compounds and therefore a greater antioxidant capacity. Consequently, in addition to determining the antioxidant capacity by conventional methods, trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorption capacity (ORAC) being the assays most commonly used to analyse this antioxidant capacity (Barba et al. 2012), it could be interesting to explore the possibility of using the determination of PPO and POD activity inhibition as an indirect measure of antioxidant capacity.

The aim of the present study was to analyse the influence of *Stevia* water extracts on the PPO and POD activities, antioxidant capacity and other bioactive compounds of a mixture of orange, mango and papaya, as a good source of antioxidant compounds, during incubation at 10 and 37 °C, in order (1) to determine the antioxidant capacity using two conventional methods, TEAC and ORAC, and, alternatively, by measuring the percentage of POD and PPO inhibition, and (2) to elucidate the potential use of *Stevia* in the food industry as a natural preservative.

Materials and Methods

Samples

Plant Material

Orange (*Citrus sinensis*, cultivar Salustiana), mango (*Mangifera indica*) and papaya (*Carica papaya*) were purchased from a local supermarket.

Crude Extract Preparation

POD and PPO were extracted using the method described by Rodrigo et al. (1996), with some modifications. A mixture of the fruit pulp (orange, mango and papaya in a proportion of 15:20:65, *w*/*w*/*w*, respectively) was homogenized in a proportion of 1:1 (*w*/*v*) with 0.05 M sodium phosphate buffer solution (Panreac Química, Barcelona, Spain) at pH 7.0 in a blender for 5 min at 4 °C. The buffer contained 1 M NaCl (Scharlau, Barcelona, Spain) and 5 % (*w*/*v*) polyvinylpolypyrrolidone (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). The homogenate was filtered through a layer of cheesecloth, and the residue was centrifuged at 20,199*g* for 30 min at 4 °C with an Avanti J-25 centrifuge (Beckman Instruments Inc., Fullerton, CA, USA.). The supernatant constituted the enzyme extract.

Stevia Infusion and Sample Preparation

Dried *Stevia* leaves were supplied by Anagalide, S. A. (Huesca, Spain). To prepare a stock solution of 8.33±0.01 % (*w*/*v*), 100 mL of boiling mineral water was added to the dried leaves (8.33 g), and the mixture was covered and allowed to infuse for 30 min. The infusion was vacuum filtered using filter paper (Whatman® no. 1), and the filtrate obtained was stored in 2-mL vials at −40 °C. Different volumes of *Stevia* stock solution (1.2, 3.6 and 6 mL) were added to 14 mL of the crude enzyme extract to obtain final *Stevia* concentrations of 0.5, 1.5 and 2.5 % (*w*/*v*), respectively. The highest *Stevia* concentration (2.5 %, *w*/*v*) was selected, taking into account the sucrose concentration of commercial fruit-based beverages and the sweetness equivalence of *Stevia* and sucrose (Savita et al. 2004). Water was added when necessary to give a final matrix volume of 20 mL. A blank sample was formulated with 14 mL of crude extract and 6 mL of water.

Methods

Determination of POD and PPO Activities

POD activity was measured spectrophotometrically, following the process reported by Rodrigo et al. (1996). PPO activity was measured by the method described by Giner et al. (2001), with some modifications. The method was based on measuring increase in absorbance at 410 nm when 1,950 μL of 0.05 M 1,2-dihydroxybenzene (pyrocatechol, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) in phosphate buffer (0.05 M, pH 7.00 with 1 M NaCl) as substrate reacted with 0.1 mL of enzyme extract.

Each enzyme determination was replicated three times at 25 °C. One unit of POD or PPO activity was expressed as one absorbance increment (at 470 and 410 nm in the conditions in which the assay was carried out, respectively) per minute and millilitre of enzyme extract. For both enzymes, the initial reaction rate was expressed as a percentage of relative activity (RA), calculated using Eq. 1:

$$\%\mathrm{RA} = 100 \cdot \left(A \middle/ A_0\right) \quad (1)$$

where A and A_0 are the current and initial POD and PPO activities, respectively.

Determination of Ascorbic Acid

A Metrohm 746 VA Trace Analyzer (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic determination. The working electrode was a Metrohm multimode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: DP50, mode DME, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential −0.10 V. The extract (5 mL) was diluted to 25 mL with the extraction solution (oxalic acid 1 % *w*/*v*, trichloroacetic acid 2 % *w*/*v*, sodium sulphate 1 % *w*/*v*). After vigorous shaking, the solution was filtered through a folded filter (Whatman® no. 1). Oxalic acid (9.5 mL) 1 % (*w*/*v*) and 2 mL of acetic acid/sodium acetate 2 M buffer (pH=4.8) were added to an aliquot of 0.5 mL of filtrate, and the solution was transferred to the polarographic cell. Determinations were done by using the peak height and standard addition method according to Barba et al. (2013).

Total Carotenoids

Extraction of total carotenoids was carried out in accordance with Lee and Castle (2001). Total carotenoids were calculated according to Ritter and Purcell (1981) using an extinction coefficient of β-carotene, $E^{1\,\%}$=2,505.

Total Phenolic Compounds

Total phenolic compounds (TPC) were determined according to the method reported by Georgé et al. (2005), with some modifications. Ten millilitres of sample was homogenized with 50 mL of a mixture of acetone/water (7/3, *v*/*v*) for 30 min. The mixture supernatants were recovered by filtration (Whatman® no. 2) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2×2 mL of distilled water. The recovered volume of the washing extract (WE) was carefully measured. To eliminate ascorbic acid, the washing extract (3 mL) was heated for 2 h at 85 °C, and this led to the heated washing extract (HWE). All extracts (RE, WE and HWE) were submitted to the Folin–Ciocalteu method, adapted and optimized (Barba et al. 2013).

Phenolic Profile

HPLC analysis was performed in accordance to Muanda et al. (2011) with some little modifications.

Total Antioxidant Capacity

TEAC Assay The method used was described by Re et al. (1999), based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$) (Sigma-Aldrich, Steinheim, Germany) compared with a standard (Trolox®) (Sigma-Aldrich, Steinheim, Germany).

ORAC Assay The ORAC assay was used, with fluorescein (FL) (Sigma-Aldrich, Steinheim, Germany) as the "fluorescent probe", described by Zulueta et al. (2009).

Statistical Analysis

Significant differences between the results were calculated by analysis of variance (ANOVA). An LSD test was applied to indicate the samples between which there were differences. A multiple regression analysis was performed to study the influence of different factors on a given parameter. Subsequently, a three-way ANOVA was applied to the results obtained to verify whether there were significant differences in the parameters studied in relation to *Stevia* percentage, incubation temperature and time, and to ascertain possible interactions between the factors. A multiple regression analysis was performed to study the influence of PPO and POD on bioactive compounds and on antioxidant capacity. All statistical analyses were performed using SPSS® (Statistical Package for the Social Sciences) v.19.0.

Results and Discussion

Effect of *S. rebaudiana* on Enzyme Activities, Antioxidant Compounds and Total Antioxidant Capacity in Fruit Extract

The results obtained for POD and PPO activities, antioxidant capacity and bioactive compounds (ascorbic acid, total carotenoids, TPC) in the fruit extract at different *Stevia* concentrations are shown in Table 1. The POD and PPO activities of the extracts were 2.31 and 7.85 ΔAbs/min mL extract in the absence of *Stevia*, respectively. The POD and PPO content of fruits and vegetables might be affected by species, cultivar and maturity. In yellow pitaya, Castro et al. (2006) found that the POD concentration was around 5.5 ΔAbs/min mL extract, and a value of 1.2 ΔAbs/min mL extract was obtained for PPO from pear (Gasull and Becerra 2006). For both enzymes, a significant decrease (p <0.05) in fruit matrix enzyme activity was observed when *Stevia* was added. However, for POD, non-significant changes were obtained with the different *Stevia* concentrations probably because a longer contact time between *Stevia* and the fruit extract would be needed for an effect to be observed.

Table 1 Enzyme activities, antioxidant compounds and total antioxidant capacity of the enzymatic fruit extract with different *S. rebaudiana* concentrations

Parameters	*Stevia rebaudiana* concentration (%, *w*/*v*)			
	0	0.5	1.5	2.5
POD[a] (ΔAbs/min mL extract)	2.31±1.41a	1.29±0.18b	1.10±0.25b	1.45±0.06b
PPO[a] (ΔAbs/min mL extract)	7.85±0.40a	6.91±0.37b	6.77±0.17b	5.49±0.13c
ORAC (mM TE)	4.72±0.54a	11.23±0.50b	30.05±0.06c	40.89±1.51d
TEAC (mM TE)	6.88±0.19a	10.84±0.43b	22.70±0.21c	34.68±0.55d
Ascorbic acid (mg/100 mL)	25.54±0.25a	25.00±0.33a	25.34±0.17a	25.27±0.12a
Total carotenoids (μg/100 mL)	247.01±10.59a	244.51±14.11a	254.49±7.06a	252.00±17.64a
Total phenolics (mg GAE/100 mL)	32.24±2.56a	86.54±2.56b	238.63±1.71c	449.16±17.08d

Different letters (a–d) in the same row indicate a significant difference as a function of the samples analysed ($p<0.05$)

POD peroxidase, *PPO* polyphenoloxidase, *ORAC* oxygen radical antioxidant capacity, *TEAC* Trolox equivalent antioxidant capacity, *TE* Trolox equivalent, *GAE* gallic acid equivalents

[a] One unit of POD and PPO activity was defined as a change in absorbance at 470 and 410 nm, respectively, per minute and millilitre of enzymatic extract

Total antioxidant capacity, measured by the TEAC and ORAC methods, was determined to establish a possible correlation with enzyme activities (POD and PPO) and with *Stevia* concentration. The antioxidant capacity values ranged from 4.72±0.54 to 40.89±1.51 and from 6.88±0.19 to 34.68 ±0.55 mM TE (millimolar Trolox equivalents) for the ORAC and TEAC assays, respectively, with a significant increase for both methods with *Stevia* concentration. Furthermore, a significant ($p<0.05$) negative correlation was found for TEAC values with POD ($r=-0.5353$) and PPO ($r=-0.5457$), respectively, and for ORAC values with POD ($r=-0.5817$) and PPO ($r=-0.5639$), respectively.

The most common antioxidant compounds found in fruit and vegetables (ascorbic acid, carotenoids and phenolic compounds) were also measured to determine the effects of *Stevia* on their concentrations as well as their correlation with enzymatic activities and antioxidant capacity. The ascorbic acid concentration in the sample without *Stevia* was 25.54± 0.25 mg/100 mL. This value is in the range of those reported by other authors when they studied the ascorbic acid concentration of papaya, mango and orange juice (USDA 2012). Furthermore, non-significant changes were found in the ascorbic acid contents of the samples with *Stevia* added [0.5–2.5 %, *w*/*v*]. An explanation for this could be that ascorbic acid is a thermolabile vitamin and was probably degraded when the *Stevia* infusion was prepared. Therefore, the ascorbic acid detected in the sample studied should come from the fruits. The carotenoid content in the fruit extract without *Stevia* was 247.01±10.59 μg/100 mL. These values were also in agreement with those previously reported by other authors in food matrices containing orange, mango and papaya (Barba et al. 2013). As in the case of ascorbic acid, non-significant changes were found between samples with and without *Stevia*. TPC ranged from 32.24±2.56 to 449.2±17.08 mg gallic acid equivalents/100 mL. TPC increased significantly according to the *Stevia* percentage, in a dose-dependent manner, reaching a maximum at 2.5 % (*w*/*v*) (Table 1). Pearson's test was done to establish possible correlations between enzyme activities, TPC and total antioxidant capacity. As was to be expected, the oxidation of phenolic substrates was catalysed by PPO, so that a significant ($p<0.05$) negative correlation was found between TPC and PPO ($r=-0.5365$), but a non-significant correlation was obtained with POD. This result could be explained by the fact that phenolic compounds are substrates mainly for PPO and, to a lesser extent, by the generation of hydrogen peroxide by POD during this oxidation (Tomás-Barberán and Espín 2001). Moreover, a positive correlation was found between TPC and TEAC ($r=0.9960$) and between TPC and ORAC ($r=0.9740$).

Effect of *S. rebaudiana* on Enzyme Activities, Antioxidant Compounds and Total Antioxidant Capacity in Fruit Extract During Incubation

Samples with [0.5–2.5 %, *w*/*v*] and without *Stevia* were incubated, and POD and PPO activities, total antioxidant capacity and TPC were determined at different time intervals, depending on the incubation conditions and *Stevia* concentration, during 10 days or, in the case of the enzymes, until their activity remained constant or below the detection limit. The incubation temperatures were 10 °C, as a refrigerated temperature that depresses enzyme activity, and 37 °C as a near-optimal temperature for PPO and POD (Dincer et al. 2002; Mohamed et al. 2008).

Independently of *Stevia* concentration, the enzyme degradation increased with incubation time (Figs. 1 and 2). Furthermore, in both enzymes, the degradation level was always higher at 37 °C than at 10 °C. In absence of *Stevia*,

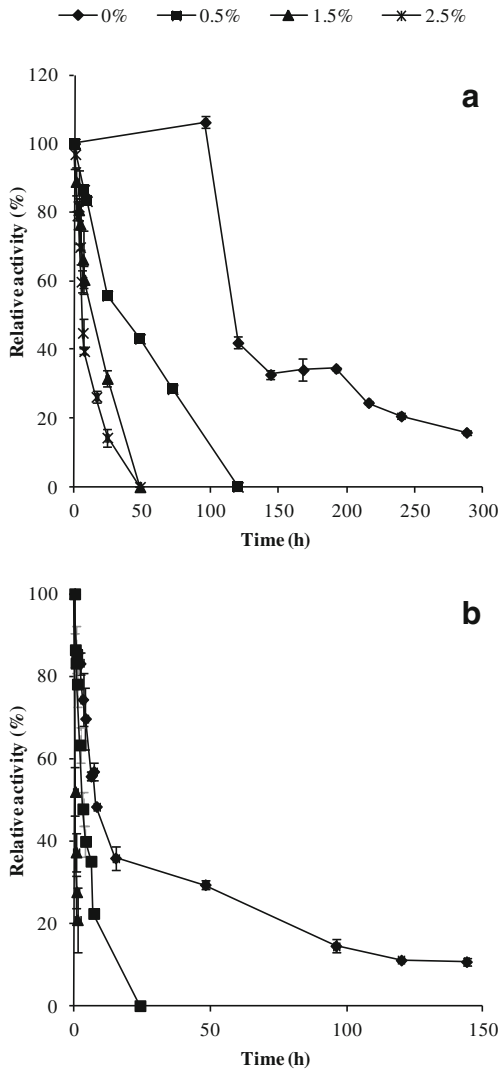

Fig. 1 Effect of *S. rebaudiana* concentration (0, 0.5, 1.5 and 2.5 %, *w*/*v*) on POD activity at different temperatures: **a** 10 °C and **b** 37 °C

the results showed that POD was more resistant to degradation, and this effect was on a larger scale at 37 °C. PPO activity was not detected after 2 days of incubation at 37 °C, while slight POD activity (around 10 %) was found after 6 days of incubation in the same conditions. In relation to PPO, activity remained constant for 2 days at 10 °C, but only for 4 h at 37 °C, decreasing to 60.4 % of the initial activity during the seventh hour of incubation, and it was not detected after the second day of incubation. On the other hand, POD activity remained stable for 4 days at 10 °C, whereas at 37 °C, it decreased to 69.7 % in only 4 h, and the activity fell by 70 % from the initial value after the second day of incubation. The relative residual activity of POD and PPO decreased as the *Stevia* concentration increased. Moreover, the higher the *Stevia* concentration assayed, the greater the activity reduction was. This decrease in activity could be attributed to an inhibitory effect of *Stevia* on the oxidative enzymes POD and PPO. The mechanism of action for *Stevia* could be explained in relation to its constituents, which include ascorbic acid (14.98 mg/100 g of extract, dry basis) and some polyphenols found in *Stevia* leaves (Muanda et al. 2011; Wölwer-Rieck 2012). It is known that PPO can be inhibited by ascorbic acid, because it reduces the quinone produced before it undergoes secondary reactions that lead to browning (Guerrero-Beltrán et al. 2005). However, it was mentioned earlier that in the present study, an increase in *Stevia* concentration did not produce a significant increase in ascorbic acid, probably because this compound suffered degradation when the *Stevia* infusion was prepared. Therefore, the inhibitory effect of *Stevia* should be attributed to some polyphenolic compounds. Plant phenolic compounds such as tocopherols, flavonoid compounds, cinnamic acid derivatives and coumarins have an antioxidant effect and therefore serve as inhibitors of PPOs, but some of them act as PPO substrates (Marshall et al. 2000). In this regard, after the analysis of the *Stevia* extract phenolic profile (data not shown), we observed a significant ($p < 0.05$) negative correlation of PPO with quercetin ($r = -0.9133$) and catechin ($r = -0.8306$), which are the major flavonoid compounds found in *Stevia* (Muanda et al. 2011). In the case of quercetin, a large number of studies in plants have demonstrated its PPO inhibitory activity (Zheng et al. 2008;

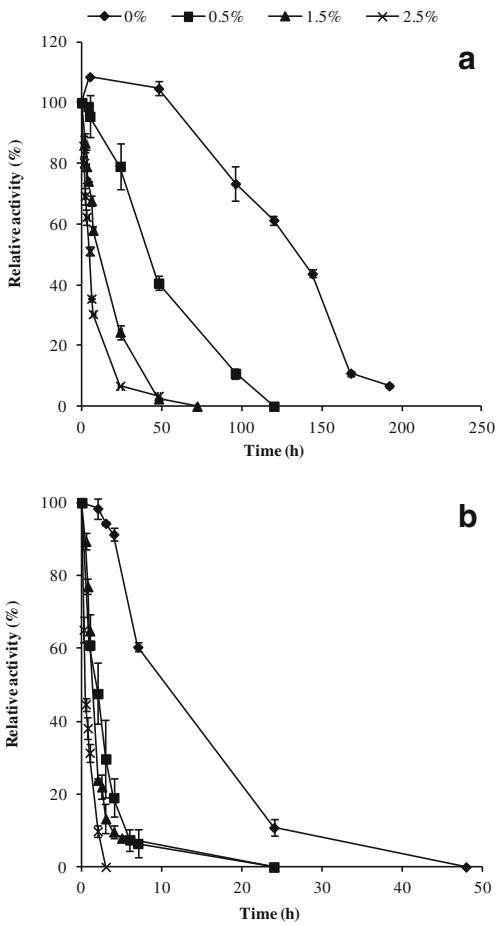

Fig. 2 Effect of *S. rebaudiana* concentration (0, 0.5, 1.5 and 2.5 %, *w*/*v*) on PPO activity at different temperatures: **a** 10 °C and **b** 37 °C

Nakashima et al. 2010). As for the catechins or catechin derivatives, they have been reported as substrates of PPO in some fruits (apple, pear, grape, peach, etc.), but they also show strong PPO inhibitory activity when they are isolated from various plants, including green tea (Nirmal and Benjakul 2009).

As for TPC, during 9 days of incubation at 10 and 37 °C, fluctuations were observed for all the samples (Fig. 3). The results obtained for the three-way ANOVA showed that incubation time, *Stevia* concentration and incubation temperature had a significant influence ($p<0.05$) on TPC, observing significant interactions between *Stevia* percentage and incubation temperature and incubation time. Figure 3 shows a reduction in TPC during the first 2 h. This decrease could be due to the action of the enzyme PPO and to a lesser extent to POD, because it coincides with the maximum values of enzyme activity. Moreover, an increase in TPC was found after 6 days of incubation at 10 and 37 °C, being significantly higher ($p<0.05$) at 37 °C and at 1.5 and 2.5 % *Stevia* (*w*/*v*). This increase could be attributed to a hypothetical formation of the Maillard reaction products via non-enzymatic browning, which can react with Folin–Ciocalteu's reagent, resulting in an overestimation of TPC (García-Alonso et al. 2009).

The antioxidant capacity of the samples with 0–2.5 % *Stevia* (*w*/*v*) measured by the TEAC and ORAC methods during incubation at 10 and 37 °C showed significant differences ($p<0.05$) between values, depending on the incubation time, *Stevia* concentration and incubation temperature. For the TEAC assay, significant interactions were found among all the three different factors studied, while for the ORAC method, a significant interaction was only obtained between *Stevia* percentage and incubation time. Overall, at the end of the incubation time, the antioxidant capacity values were higher when the incubation temperature was 37 °C, except for the sample without addition of *Stevia*. This can be attributed to an increase in the

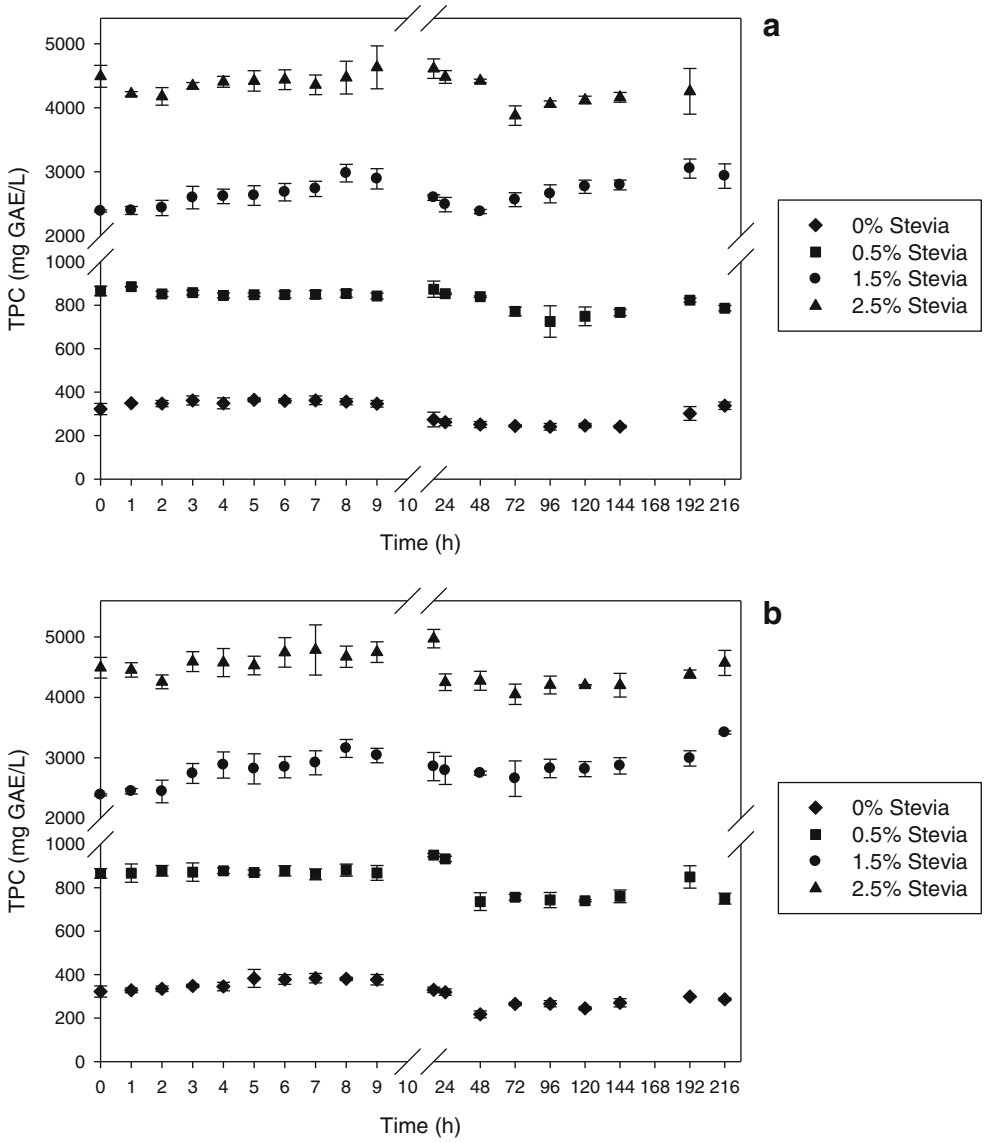

Fig. 3 Total phenolic content (TPC) modifications during incubation at **a** 10 °C and **b** 37 °C

extractability of the antioxidant compounds found in *Stevia*. In addition, the formation of new compounds with antioxidant capacity, such as melanoidins, derived from the Maillard reaction, which are able to scavenge free radicals (Zulueta et al. 2012), could also be responsible for the increase in the TEAC and ORAC sample values during incubation at 37 °C.

In the present study, so far, it has been shown that an increase in *Stevia* concentration inhibits enzyme activity to a certain extent and increases TPC and antioxidant capacity. Thus, a remarkable antioxidant effect of *Stevia* has been demonstrated. To maintain the antioxidant capacity of a matrix, it is necessary to reduce the activity of oxidative enzymes. Therefore, given that the ORAC and TEAC methods are used to determine the antioxidant capacity, it would also be useful to determine to what extent the compounds contributing to the antioxidant capacity, including polyphenols, are affected, by measuring the activity of the enzymes involved in their degradation. To evaluate the relationship between the total antioxidant capacity measured by the TEAC and ORAC methods and the enzyme activities, a multivariate regression analysis was performed (Eqs. 2–3) (the results are shown in the significant cases, $p<0.05$).

$$\mathrm{PPO} = 5.332 - 0.086 \cdot \mathrm{TEAC}. \quad (2)$$

$$\mathrm{POD} = 1.588 - 0.027 \cdot \mathrm{TEAC}. \quad (3)$$

As can be observed, ORAC values did not contribute significantly ($p>0.05$) to explain the POD and PPO modifications. Matrices rich in hydrophilic antioxidants react quickly with the ABTS radical, and this action is favoured by the TEAC method because it is a redox reaction. Moreover, as the ORAC method is a reaction based on the transfer of H atoms, these groups present in this matrix are more stable, so ORAC was not greatly modified during incubation (Zulueta et al. 2012). Therefore, in accordance with the results, for this matrix, it seems more appropriate to measure antioxidant capacity with the TEAC method and consequently to establish the correlation between POD and PPO with the TEAC method. As for the relationship (Eqs. 2–3), a negative significant dependence ($p<0.05$) during incubation was obtained between enzyme activity and TEAC values, meaning that antioxidant capacity increases when enzyme activity decreases. This dependence was higher for PPO than for POD, as indicated by the equation coefficients (0.086 and 0.027 for PPO and POD, respectively) and corroborated by the fact that reactions catalysed by PODs depend on the previous action of PPOs, because PODs use the hydrogen peroxide generated during the oxidation of phenolic compounds by PPOs.

Conclusions

In the present study, *Stevia* has shown an antioxidant activity based on the inhibition of oxidative enzymes and on the increase in TPC and antioxidant capacity. Furthermore, a relationship has been found between enzyme activity and antioxidant capacity, which could result in the use of the measurement of enzyme activity as an indirect index of sample antioxidant capacity. In this regard, the use of substances that, besides acting as ingredients in the food formulation, perform some functional role (antioxidants or antimicrobials, for example) is of increasing interest to food processors. The potential use of these natural "green" *Stevia* extracts could offer the food processing industry an application not only as an alternative sweetener but also as a natural preservative.

Acknowledgments The authors thank the Interministerial Commission for Science and Innovation (Spain) for its support through project number AGL2010-22206-C02-01ALI. Criado, M.N. thanks the CSIC for the award of a JAE-Tec contract. Barba, F.J. received a contract of employment from this project to carry out the study. Finally, the authors also thank the Anagalide company (Barbastro, Huesca, Spain) for providing dried *Stevia* leaves.

References

Barba, F. J., Cortés, C., Esteve, M. J., & Frígola, A. (2012). Study of antioxidant capacity and quality parameters in an orange juice–milk beverage after high-pressure processing treatment. *Food and Bioprocess Technology, 5*(6), 2222–2232.

Barba, F. J., Esteve, M. J., Tedeschi, P., Brandolini, V., & Frígola, A. (2013). A comparative study of the analysis of antioxidant activities of liquid foods employing spectrophotometric, fluorometric, and chemiluminescent methods. *Food Analytical Methods, 6*(1), 317–327.

Castro, J. A., Baquero, L. E., & Narváez, C. E. (2006). Catalase, peroxidase and polyphenoloxidase from pitahaya amarilla fruits (*Acanthocereus pitajaya*). *Revista Colombiana de Química, 35*(1), 91–101.

Chen, L., Mehta, A., Berenbaum, M., Zangeri, A. R., & Engeseth, N. J. (2000). Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. *Journal of Agricultural and Food Chemistry, 48*, 4997–5000.

Dincer, B., Colak, A., Aydin, N., Kadioglu, A., & Güner, S. (2002). Characterization of polyphenoloxidase from medlar fruits (*Mespilus germanica* L., Rosaceae). *Food Chemistry, 77*, 1–7.

El Nehir, S., & Simsek, S. (2012). Food technological applications for optimal nutrition: an overview of opportunities for the food industry. *Comprehensive Reviews in Food Science and Food Safety, 11*, 2–12.

Commission Regulation (EU) No 1131/2011 of November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council with regard to steviol glycosides. Official Journal of the European Union.

García-Alonso, F. J., Bravo, S., Casas, J., Pérez-Conesa, D., Jacob, K., & Periago, M. J. (2009). Changes in antioxidant compounds during the shelf life of commercial tomato juices in different packaging materials. *Journal of Agricultural and Food Chemistry, 57*, 6815–6822.

Gasull, E., & Becerra, D. (2006). Characterization of polyphenoloxidase extracted from pears (cv. Packam's Triumph) and apples (cv. Red Delicious). *Información Tecnológica, 17*(6), 69–74.

Georgé, S., Brat, P., Alter, P., & Amiot, M. J. (2005). Rapid determination of polyphenols and vitamin C in plant-derived products. *Journal of Agricultural and Food Chemistry, 53*, 1370–1373.

Giner, J., Gimeno, V., Barbosa-Cánovas, G. V., & Martín, O. (2001). Effects of pulsed electric field processing on apple and pear

polyphenoloxidases. *Food Science and Technology International, 7*(4), 339–345.

Guerrero-Beltrán, J. A., Swanson, B. G., & Barbosa-Cánovas, G. V. (2005). Inhibition of polyphenoloxidase in mango puree with 4-hexylresorcinol, cysteine and ascorbic acid. *LWT-Food Science and Technology, 38*(6), 625–630.

Kim, I. S., Yang, M., Lee, O. H., & Kang, S. N. (2011). The antioxidant activity and the bioactive compound content of *Stevia rebaudiana* water extracts. *Food Science and Technology, 44*, 1328–1332.

Lee, H. S., & Castle, W. S. (2001). Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd blood orange juices. *Journal Agricultural and Food Chemistry, 49*, 877–882.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry, 132*, 1121–1132.

Loizzo, M. R., Tundis, R., & Menichini, F. (2012). Natural and synthetic tyrosinase inhibitors as antibrowning agents: an update. *Comprehensive Reviews in Food Science and Food Safety, 11*, 378–398.

Marshall MR, Kim JM, Wei CI (2000) Enzymatic browning in fruits, vegetables and seafoods. FAO. http://www.fao.org/ag/ags/agsi/ENZYMEFINAL/Enzymatic%20Browning.html. Accessed 12 November 2012

Mohamed, S. A., El-Badry, M. O., Drees, E. A., & Fahmy, A. S. (2008). Properties of a cationic peroxidase from *Citrus jambhiri* cv. Adalia. *Applied Biochemistry and Biotechnology, 150*, 127–137.

Muanda, F. N., Soulimani, R., Diop, B., & Dicko, A. (2011). Study on chemical composition and biological activities of essential oil and extracts from *Stevia rebaudiana* Bertoni leaves. *LWT-Food Science and Technology, 44*, 1865–1872.

Nakashima, S., Matsuda, H., Oda, Y., Nakamura, S., Xu, R., & Yoshikawa, M. (2010). Melanogenesis inhibitors from the desert plant *Anastatica hierochuntica* in B16 melanoma cells. *Bioorganic Medicinal Chemistry, 18*, 2337–2345.

Nirmal, N. P., & Benjakul, S. (2009). Melanosis and quality changes of pacific white shrimp (*Litopenaeus vannamei*) treated with catechin during iced storage. *Journal of Agricultural and Food Chemistry, 57*, 3578–3586.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237.

Ritter, E. D., & Purcell, A. E. (1981). Carotenoid analytical methods. In X. Bauernfeind (Ed.), *Carotenoids as colorants and vitamin A precursors* (pp. 815–923). New York: Academic.

Rodrigo, C., Rodrigo, M., Alvarruiz, A., & Frígola, A. (1996). Thermal inactivation at high temperatures and regeneration of green asparagus peroxidase. *Journal of Food Protection, 59*(10), 1065–1071.

Savita, S. M., Sheela, K., Sunanda, S., Shankar, A. G., Ramakrishna, P., & Sakey, S. (2004). Health implications of *Stevia rebaudiana*. *Journal of Human Ecology, 15*(3), 191–194.

Tadhani, M. B., Patel, V. H., & Subhash, R. (2007). In vitro antioxidant activities of *Stevia rebaudiana* leaves and callus. *Journal of Food Composition and Analysis, 20*, 323–329.

Tomás-Barberán, F. A., & Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture, 81*(9), 853–876.

USDA (2012). Nutrient database for standard reference. From the Nutrient Data Laboratory Home Page: http://www.nal.usda.gov/ fnic/foodcomp/search/. Accessed 20 Mar 2013.

Wölwer-Rieck, U. (2012). The leaves of *Stevia rebaudiana* (Bertoni), their constituents and the analyses thereof: a review. *Journal of Agricultural and Food Chemistry, 60*, 886–895.

Zheng, Z., Cheng, K. W., Chao, J., Wu, J., & Wang, M. (2008). Tyrosinase inhibitors from paper mulberry (*Broussonetia papyrifera*). *Food Chemistry, 106*, 529–535.

Zulueta, A., Esteve, M. J., & Frígola, A. (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry, 114*(1), 310–316.

Zulueta, A., Barba, F. J., Esteve, M. J., & Frígola, A. (2012). Changes in quality and nutritional parameters during refrigerated storage of an orange juice-milk beverage treated by equivalent thermal and non-thermal processes for mild pasteurization. *Food and Bioprocess Technology*. doi:10.1007/s11947-012-0858-x.