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Isolation and thermostability of peroxidase isoenzymes from apple cultivars Gala and Fuji

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Abstract

Extracts of soluble peroxidase and ionically-bound peroxidase were obtained from the peel and pulp of apples (*Mallus comunis*), cultivars Gala and Fuji, using 100 mM sodium phosphate buffer and pH 6.0 and 7.0 for pulp and peel of Gala cultivar, respectively and pH 5.5 for the pulp and the peel of cultivar Fuji. Then the enzymatic activity of peroxidase (POD) was determined in both extracts, soluble fractions and ionically-bound. Electrophoresis results shows similar compositions for the anionic and cationic isoenzymes in both cultivars. The molecular weights and p*I* values of the isolated isoenzymes from the Fuji cultivar were: two anionic, A_1 and A_2 (38, 28 kDa and p*I* 4.4; 5.0, respectively) and three cationic C_1, C_2 and C_3 (40, 34, 26 kDa and p*I* 8.0, 8.3, 9.0, respectively). From the Gala cultivar, also, two anionic isoenzymes were isolated A_2 and A_4 (28, 26 kDa and p*I* 5.0 and 5.7, respectively) and three cationic isoenzymes C_1, C_2 and C_3 (40, 34, 26 kDa and p*I* 8.0, 8.3 and 9.0, respectively). The cationic isoenzymes were more heat-stable under the heat treatments at 65, 70, 75 and 80 °C in both cultivars.

Keywords: Apple; *Mallus comunis*; Peroxidase; Isoenzymes; Isolation; Thermostability

1. Introduction

Enzymatic browning of raw fruits and vegetables is mainly due to the oxidation of natural phenolic compounds into quinones that, in turn, are polymerized to brown, red or black pigments. The control of the activity of the peroxidase is important in the preservation and in the processing of foods, otherwise it could promote darkening in fruits and vegetables and their marketed products. These enzymes can participate in a great number of oxidative reactions, such as, colour change, degradation of chlorophyll or auximas, oxidation of phenols, oxidation of indole acetic acid and biosynthesis of lignin, and many of these factors are also associated with the flavour, colour, texture and nutritional qualities of foods (Clemente & Pastore, 1998). Peroxidase is also intimately related to flavour loss and odour of stored foods, as well as a great variety of biodegradation reactions (Clemente & Robinson, 1995; Clemente, 1996). Peroxidase accumulation in the peel can be related to development to physiological disorders (Du & Bramlage, 1995). During the process of ripening, post-harvesting and freezing of fruits, modification is observed in the pulp with development of off-flavor (Cano, Ancos, & Lobo, 1995). Apples are commonly stored for long periods at low temperature, and during this time disorders can develop. Peroxidation is a prominent factor in apple senescence (Du & Bramlage, 1995). In fruit juice production, some peel is included, and this contributes to the increase of the activity of the peroxidase (Clemente, 1993). The extracts prepared from plants to verify peroxidase activity show that this enzyme may be in soluble form or ionic form, linked to the cellular wall (Clemente, 1993; Moulding, Grant, McLellan, & Robinson, 1987).

The commercial heat treatments used for processing fruit juice and vegetables, for instance high temperature for short time (HTST), is not very effective for irreversible inactivation of the peroxidase (Khan & Robinson, 1993; McLellan & Robinson, 1987).

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The loss of the activity of peroxidase during thermal treatment is dependent on the origin of the enzyme, pH, enzymatic concentration and assay methods. Peroxidase inactivation in extracts of plants have been shown, in a general way, to be non-linear regarding the factors time and temperature. This is possibly due to the presence of isoperoxidases with different degrees of thermostability. In general, it is accepted that peroxidase, unlike other enzymes which are inactivated by heat, remains active. This enzyme is not totally inactivated and, can contribute to the loss of acceptibility and the development of unpleasant flavours in foods (Clemente, 1996; Khan & Robinson, 1993; Lu & Whitaker, 1974). Our general objective was to study POD isoenzymes isolated from apple fruits and their behaviour upon thermal treatment.

2. Materials and methods

2.1. Materials

Apples (cv. Gala and Fuji) were harvested ripe and brought into the food biochemistry laboratory 24 h after the harvest. The fruits supplied were made by CELPLAC Company from Guarapuava-PR – Brazil. On arrival, undamaged fruits, free from infection were selected for the assays. All of the chemical products used were of analytical grade, obtained from BDH Light Laboratories Ltd.

2.2. Extracts preparation

Prior to extracting peroxidases from apple, the optimum pH for extracting these enzymes from pulp and peel was determined. The crude extracts of peroxidase were prepared using 100 g of peel and 300 g of pulp from each apple cultivar. The samples were homogenized separately in a blender with 100 and 300 ml, respectively, of cold sodium phosphate buffer (100 mM pH 5.5 for pulp and peel of the apple Fuji, pH 6.0 for pulp of the apple Gala and pH 7.0 for peel of the apple Gala). After homogenization the samples were filtered through cotton tissue and the filtrates were centrifuged to 17,000*g* for 20 min, at 4 °C. The supernatant collected was designated ''soluble peroxidase'' and stored at −18 °C. For the extraction of the ionically-bound peroxidase, the residue remaining after the extraction of the soluble peroxidase fraction was resuspended in 100 ml 1 M of NaCl in sodium phosphate buffer (100 mM pH as described above), and centrifuged as described above. The supernatant collected was stored at −18 °C.

2.3. Analytical methods

2.3.1. Concentration of enzymatic extracts

The crude enzymatic extracts from peel and pulp were concentrated by precipitation of the proteins using cold acetone, in the proportion of 2 to 1 (acetone/extract). The precipitate was resuspended in sodium phosphate buffer (100 mM and same pH as extract was) in a volume five times smaller in relation to the initial volume of the extract.

2.3.2. Enzymatic activity

The method used to determine peroxidase activity of POD was based on the method described by Clemente (1998). The reaction mixture contained 2.7 ml of 0.03% H_2O_2 in 100 mM sodium phosphate buffer at pH 6.0 and 0.2 ml of the peroxidase extract. The enzymatic reaction was initiated by addition of 0.1 ml, 1% (w/v) *o*-dianisidine solution in methanol and the initial change in absorbance was recorded at 460 nm at 25 °C using UV-VILE spectrophotometer (Hitachi U-200) for a period of 1 min. Each sample was assayed in triplicate. A unit of activity of POD was defined as the increase of a unit of absorbance per min/g of sample.

2.3.3. Gel filtration chromatography

Before application in the chromatographic column, the samples were dialyzed overnight in sodium phosphate buffer (10 mM, pH 7.0) at 7 °C following a filtration using a micro filter (0.22 μm) to prevent small particles blocking the filters in the column. A column (16 mm i.d. × 70 cm) was packed with gel (Sephacryl S-100-HR, marks Sigma) and pre-equilibrated with 100 mM phosphate buffer, pH 6.0, as the eluent. A 5 ml sample of the concentrated and dialyzed fraction was applied to the column. The column was run at flow rate 30 ml h^{-1}; 5 ml fractions were collected and assayed for POD activity and protein content. The isoenzymes in each fraction were visualized, by electrophoresis and staining for POD activity.

2.3.4. Electrophoresis

2.3.4.1. Peroxidase. The method used was as described by Alvim and Clemente (1998) to identify the composition of the isoenzymes in the concentrated extracts. The concentrated extracts were absorbed on filter paper Whatman number 3 (5×6 mm) and inserted ver-

Table 1
Peroxidase activity in the apples cultivars Gala and Fuji ($n = 3$)

Extracts	POD units ± δ
Soluble fraction from pulp (Fuji)	8470 ± 0.020
Soluble fraction from peel (Fuji)	9585 ± 0.015
Ionically-bound fraction from pulp (Fuji)	2130 ± 0.020
Ionically-bound fraction from peel (Fuji)	3305 ± 0.010
Soluble fraction from pulp (Gala)	0960 ± 0.020
Soluble fraction from peel (Gala)	1090 ± 0.010
Ionically-bound fraction from pulp (Gala)	1175 ± 0.010
Ionically-bound fraction from peel (Gala)	1745 ± 0.010

n = number of repetitions, δ = standard deviation.

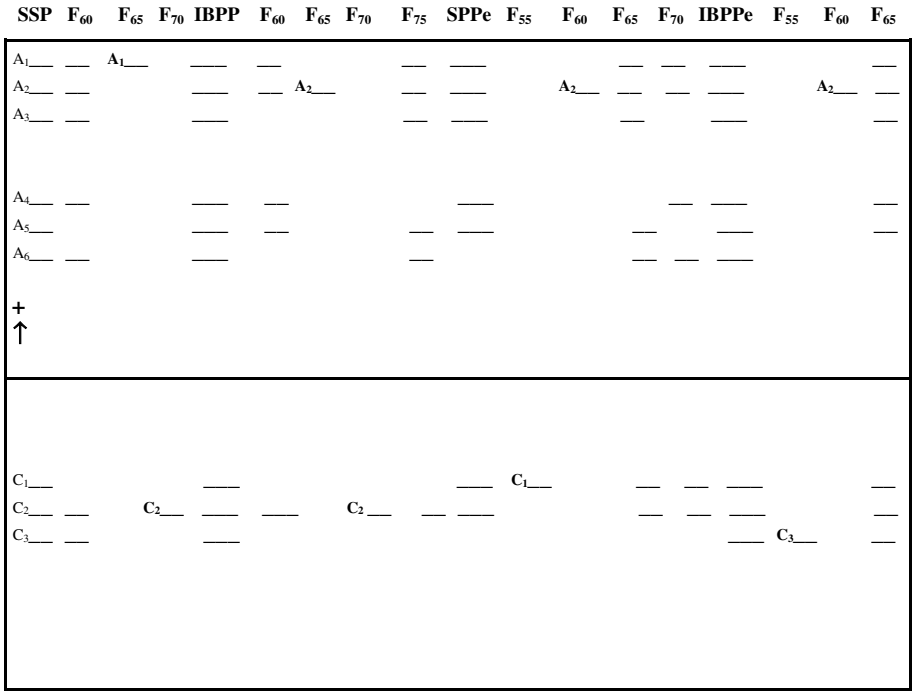

Fig. 1. Electrophoresis patterns obtained for the fractions after the fractionation on a Sephecyl HS 100 column (Fuji cultivar). SSP = soluble peroxidase from pulp; IBPP = ionically-bound peroxidase from pulp; SPPe = soluble peroxidase from peel; IBPPe = ionically-bound peroxidase from peel; F = fractions carried out from the column.

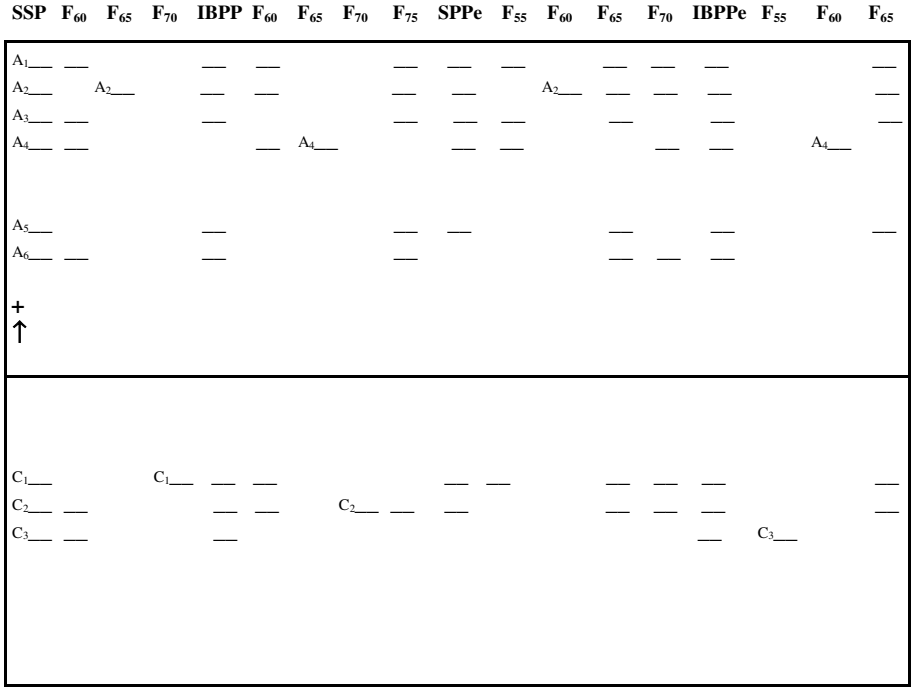

Fig. 2. Electrophoresis patterns obtained for the fractions after the fractionation on a Sephecyl HS 100 column (Gala cultivar). SSP = soluble peroxidase from pulp; IBPP = ionically-bound peroxidase from pulp; SPPe = soluble peroxidase from peel; IBPPe = ionically-bound peroxidase from peel; F = fractions carried out from the column.

tically in a gel of starch to 10%, prepared with buffer solution of tris/citric acid and buffer solution of boric acid/lithium hydroxide, in the proportion of 9:1. Buffer solution of boric acid/lithium hydroxide (pH 8.3) was used in the chambers. After the protein migration, the gel was incubated 37 °C, in a light shelter. The gels

were stained using 50 ml of sodium phosphate buffer solution pH 6.0, 10 ml of methanol and 2.5 ml of 1% solution of *o*-dianisidine (w/v) in methanol. After a period of 5 min, 8 ml of a solution hydrogen peroxide were added, to give the colouration. The stained gels were washed with distilled water and fixed with solution containing distilled water, methanol and acetic acid (5:5:1).

2.3.5. Protein determination

Total protein content of the extract was estimated according to Bradford (1976) and also estimated at 280 nm.

2.3.6. Thermostability

The concentrated enzymatic extracts of the peel and pulp were submitted to thermal treatments at 65, 70, 75, 80 °C, for periods of 1–10 min and the residual activity of POD was determined as described previously.

3. Results and discussion

3.1. Enzymatic activity

Table 1 shows the peroxidase activity in the enzymatic extracts of the peel and pulp of the apple cultivars Gala and Fuji. It was verified that the higher peroxidase activity was present in the extracts from the peels, in both cultivars. The results accord with the results reported by Richard and Nicolas (1989), who found higher activity of peroxidase in the peel and Alvim and Clemente (1998) who reported a higher enzymatic activity for soluble and inonically bound peroxidase fractions extracted from tangerine peel.

After the concentrated extracts were obtained from the filtration processes, dialysis and gel chromatography (Sephacryl S-100-HR), the fractions with enzymatic activity were used for electrophoresis assay. The results of electrophoresis are illustrated in Figs. 1 and 2.There are different compositions of isoenzymes (cationic and anionic), similar to the result found by Valderrama, Marangoni, and Clemente (2001).

The fraction F_{65} from SSP is the anionic isoenzyme A_1 and the fractions F_{60} from SPPe and F_{60} from IBPPe the anionic isoenzyme A_2 for the cultivar Fuji. Patterns of cationic isoenzymes were observed in the extracts from the cultivar Fuji: the isoenzyme (C_2) was observed in fraction F_{70} from SSP and also fraction F_{70} from IBPP. In the fractions F_{55} from SPPe and F_{55} from IBPPe, cationic isoenzymes C1 and C3, respectively, were observed. In the cultivar Gala, the composition of anionic isoenzymes was verified (A_2) in the fractions F_{65} from SSP and F_{60} from SPPe, and in the fractions F_{65} from IBPP and F_{60} from IBPPe the same anionic isoenzyme was present (A_4). From the extracts of the cultivar Gala, cationic isoenzyme (C_1) was observed in the fractions F_{70} from SSP and $F_{55}$from SPPe, (C_2) in fraction F_{70} from IBPP and (C_3) in fraction F_{55} from IBPPe.

The results of electrophoresis assay for determination of the molecular weight and p*I* of the isolated isoenzymes presented the following results: anionic isoenzymes, A_1, A_2 and A_4, 38, 28 and 26 kDa and 4.4, 5.0 and 5.7, respectively, and for the cationic isoenzymes, C_1, C_2 and C_3, 40, 34 and 26 kDa and 8.0, 8.3 and 9.0, respectively. These results for the molecular weights of the isoenzymes are similar to those reported in the literature (Clemente, 1998; Clemente, 2002; Khan & Robinson, 1993; Prestamo, 1989; Silva, Lourenço, & Neves, 1990).

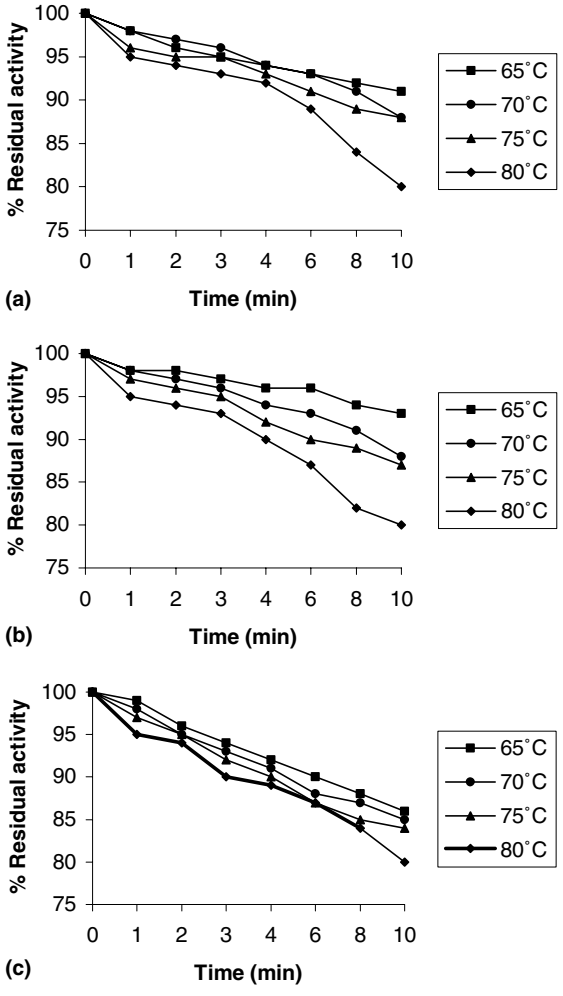

Fig. 3. Residual activity of the cationic peroxidase isoenzymes C_1 (a); C_2 (b) and C_3 (c) from Fuji cultivar.

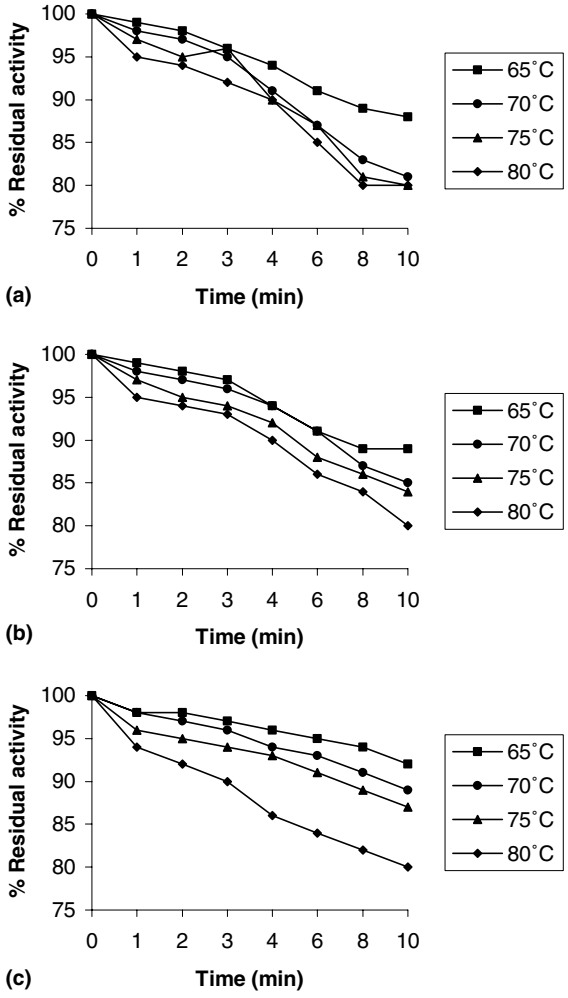

Fig. 4. Residual activity of cationic peroxidase isoenzymes C_1 (a); C_2 (b) and C_3(c) from Gala cultivar.

During the thermal treatment of the isolated isoenzymes, an almost continuous decrease of peroxidase activity was observed at all temperatures. The variations of the enzymatic activity for the isoenzymes from pulp and peel, as functions of the thermal treatment at 65, 70, 75 and 80 °C are illustrated in Figs. 3–6.

The thermal treatment of the peroxidase proved to be non-linear in relation to the factors time and temperature. These results are in agreement with the results reported by McLellan and Robinson (1984) in their work with peroxidase in orange. Khan and Robinson (1993) also verified this type of behaviour in their study of thermostability of mango peroxidase.

The peroxidase was not inactivated at the tested temperatures. In both the soluble fractions, as the ionic fractions, the residual activity of the cationic isoenzyme of the peroxidase is at least 80% (a maximum inactivation of only 20%). The residual activity of the anionic isoenzyme of the peroxidase is at least 30%, indicating a maximum inactivation of 70% in the soluble fraction and in the ionically-bound fraction.

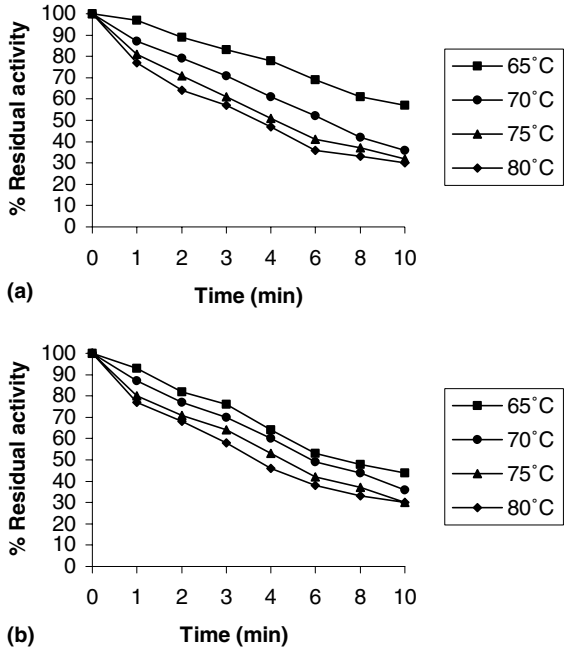

Fig. 5. Residual activity of the anionic peroxidase isoenzymes A_1 (a) and A_2 (b) from Fuji cultivar.

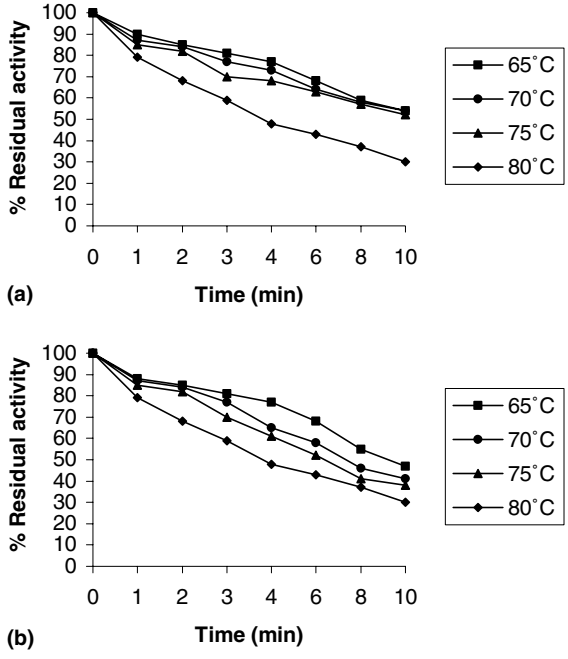

Fig. 6. Residual activity of the anionic peroxidase isoenzymes A_2 (a) and A_4 (b) from Gala cultivar.

4. Conclusions

With the determination of the enzymatic activity of peroxidase in the crude extract, it may be concluded that, the peels of the cultivars Gala and Fuji have the most activity.

The isolation of isoperoxidases from soluble and ionic fractions of the peel and pulp, was confirmed through the electrophoresis, similar compositions of anionic and cationic isoenzymes being observed both cultivars, however, some differences were observed in the p*I*.

Electrophoretic assays for determination of the molecular weight and p*I* of the isolated isoenzymes were similar to peroxidases in other types of fruits.

The thermostability of the isolated isoenzymes of the peroxidase, showed non-linear activity of POD in relation to the factors time and temperature, where it concluded that isoenzymes exist with different thermostabilities. The conditions used in the thermal treatment were severe enough to promote a maximum inactivation of 70% for the anionic isoenzymes isolated from the peel and pulp of the cultivars Gala and Fuji. However the same treatment applied to the isolated cationic isoenzymes showed a maximum inactivation of only 20%. The loss of the activity of these isoenzymes during the thermal treatment is associated with the origin of the enzyme, pH, enzymatic concentration, assay methods and possibly other species of peroxidase with different amino acid sequences. In general, it is accepted that POD, unlike other enzymes that are inactivated by heat, remain active and may contribute to the loss of acceptibility and development of unpleasant flavours in foods.

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