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Heat Stability of Peroxidases from Orange

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ABSTRACT

Soluble and ionically bound peroxidases have been obtained from orange juice and the albedo. A high level of peroxidase activity was present in the albedo. Non-linear heat inactivation was observed for orange peroxidases. The bound peroxidases were particularly heat stable and regenerated when held at 30°C following heat inactivation. A small amount of regeneration was observed for orange soluble peroxidases.

INTRODUCTION

Peroxidase has long been known to be a highly heat stable enzyme (Burnette, 1977); indeed, it is considered that any heat treatment sufficient to destroy peroxidase activity will also destroy most other enzyme systems (Reed, 1975). Peroxidase is an oxidoreductase enzyme capable of catalysing a large number of oxidation reactions in plants using either peroxides, or, in some cases, oxygen, as a hydrogen acceptor. Any residual peroxidase in blanched vegetable or fruit products could therefore be a contributory factor to quality deterioration caused by oxidative reactions. Correlations have been reported between peroxidase activity and off-flavours in some vegetables (Haard, 1977); for example, Brussels sprouts (Steinbuch *et al.*, 1979), and corn-on-the-cob (Lee &

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Hammes, 1979). In the case of oranges, more severe extraction procedures have resulted in higher levels of peroxidase in the juice and associated loss of flavour quality (Bruemmer *et al.*, 1976).

The heat inactivation of peroxidase in vegetable extracts is a biphasic process (Yamamoto *et al.*, 1962; Duden *et al.*, 1975; Mihalyi & Vamos-Vigyazo, 1975). A large decrease in activity is observed during the initial stages of a given heating process, but the rate of inactivation then changes to a much slower process. Regeneration of peroxidase following heat inactivation has also been reported for some vegetable extracts, e.g. kohlrabi (Vamos-Vigyazo *et al.*, 1979), Brussels sprouts and cabbage (McLellan & Robinson, 1981) and for purified horseradish peroxidase (Lu & Whitaker, 1974). Longer heat treatment was found to be necessary in order to irreversibly inactivate horseradish peroxidase than that required for reversible inactivation (Adams, 1978). Hence HTST treatments commonly used commercially in fruit and vegetable processing are less effective for irreversible peroxidase inactivation than the traditional, more prolonged, methods (Adams, 1978). Pasteurisation of fruit juice often involves a flash point method, with temperatures of approximately 90 °C maintained for only 30–60 s. It is doubtful whether such high temperature–short time heat treatment will destroy all peroxidase activity.

Commercial fruit juice production includes some of the albedo or pith of the fruit in the juice. This causes the juice to be slightly viscous due to the high pectin content of the pith. Peroxidase levels have been reported to be higher in orange pith than in orange juice (Bruemmer *et al.*, 1976) so any pith included in orange juice will make a large contribution to the total peroxidase activity. The current investigation includes consideration of the heat stability of orange pith peroxidase as well as orange juice peroxidase.

MATERIALS

o-Dianisidine was purchased from Koch Light Laboratories Ltd, Colnbrook, Bucks., and pentosanase 36L from Novo Enzyme Products Ltd, Windsor, Berks. All other chemicals were obtained in the analar grade from BDH Chemicals Ltd, Poole, Dorset, Great Britain.

Oranges, *Citrus autantium*, were purchased in a local supermarket.

METHODS

Extraction of peroxidases

For the extraction of peroxidases from the juice of oranges, the fruit was cut in half and squeezed by hand. The juice obtained (60 ml) was then centrifuged at 15000 *g* for 20 min at 4 °C. The supernatant was concentrated to 50 % of its original volume using an Amicon concentrator with a PM10 membrane, and then dialysed against 0·01 M sodium phosphate buffer at pH 6·0. This fraction of peroxidase activity was designated the juice soluble peroxidase fraction (JSP) and was held at −18 °C until required for assay. The residue remaining following centrifugation of the orange juice was washed twice by resuspending in a total volume of 60 ml 0·01 M sodium phosphate buffer, pH 6·0, and then centrifuging as before. The final residue was considered to be free of soluble peroxidase activity and was resuspended in 60 ml 1 M NaCl and centrifuged at 15000 *g* for 20 min at 4 °C. The supernatant fluids were termed the 'juice ionically bound peroxidase fraction' (JIP) and were stored at −18 °C until required for assay.

For the extraction of peroxidases from the albedo or pith of the orange, first the albedo was carefully separated from the endocarp and flavedo. The albedo (10 g) was then homogenised for 1 min in 100 ml 0·01 M phosphate buffer at pH 6·0 containing 1 % (v/v) pentosanase, using a Waring blender. The presence of pentosanase prevented gelling of the albedo extracts due to the high pectin content of this fraction. The resulting suspension of orange albedo in phosphate buffer was centrifuged at 15000 *g* for 20 min at 4 °C, and the supernatants, designated the 'albedo soluble peroxidase fraction' (ASP), were collected and stored at −18 °C. The residue was washed twice to remove any remaining soluble peroxidase activity by resuspending in 0·01 M phosphate buffer, pH 6·0 (100 ml) centrifuging and discarding the supernatant. The washed residue was finally resuspended in 100 ml 1 M NaCl, centrifuged as before and the supernatant fluids collected and stored at −18 °C. This fraction was termed the 'albedo ionically bound peroxidase' (AIP). Both albedo fractions were diluted 1 in 10 with 0·01 M phosphate buffer before assay.

Inactivation and regeneration of peroxidase activity

Heat treatments leading to the inactivation of peroxidase were carried out at 60, 65, 70 and 75 °C for up to 10 min. The regeneration of heat

inactivated peroxidases was allowed for up to 2·5 h at 30 °C. The chosen conditions of heat treatment prior to regeneration measurements were different for each fraction of peroxidase so as to inactivate a similar proportion of the total peroxidase activity in each case. Some 80% inactivation was achieved for JSP, ASP and AIP by heating at 75 °C for 4 min, 70 °C for 4 min and 75 °C for 8 min, respectively. The more stable JIP fraction only showed 50% inactivation in spite of heating at 75 °C for 8 min.

The experimental details for both inactivation and regeneration procedures have been given elsewhere (McLellan & Robinson, 1981). Each time/temperature treatment was carried out on triplicate samples.

Assay of peroxidase activity

Peroxidase activity was estimated using the *o*-dianisidine method described previously (McLellan & Robinson, 1981). Initial reaction rates were measured using a Pye Unicam SP 8200 UV/Vis Spectrophotometer.

RESULTS AND DISCUSSION

Table 1 shows the peroxidase activity found in extracts of orange and albedo. The change in absorbance per minute per millilitre of extract was less than 0·4 for both soluble and ionically bound fractions of orange juice. The albedo fractions, however, were found to contain more than twenty times this level of peroxidase activity, in extracts obtained following extraction of 10 g albedo in 100 ml buffer. Furthermore, the presence of pentosanase in albedo soluble extracts may have reduced the measured peroxidase activity. The addition of pentosanase to juice soluble extracts resulted in a reduction of more than 50% in the peroxidase activity detected (Cruz Para, 1982).

The high activity for peroxidase measured in the extracts of orange albedo suggests that, in the commercial production of orange juice, the small amounts of albedo which are included in juice may considerably increase the levels of peroxidase activity present. Bruemmer *et al.* (1976) stated that more severe extraction procedures resulted in higher peroxidase levels which, in turn, were associated with poor quality. This high level of peroxidase activity may have been due to a greater proportion of albedo peroxidase being released into the juice. It is

TABLE 1
Peroxidase Activity in Orange Extracts

Extract	ΔA_{460} (min^{-1} ml^{-1})
Juice soluble peroxidase (JSP)	0·337
Juice ionically bound peroxidase (JIP)	0·229
Albedo soluble peroxidase (ASP)	8·815
Albedo ionically bound peroxidase (AIP)	8·415

therefore relevant to include a study of albedo peroxidase heat stability when considering possible contributory factors to the quality of stored orange juice.

During the assay of peroxidase in juice soluble extracts a lag period was observed between the addition of reagents and the oxidation of the substrate, *o*-dianisidine, as measured by a change in absorbance at 460 nm. The lag period could be eliminated by dialysis of the juice prior to the estimation of peroxidase activity and is therefore believed to have been caused by a low molecular weight, water-soluble component of the juice, possibly ascorbic acid (Bruemmer *et al.*, 1976).

Inactivation plots for soluble and bound peroxidases from orange juice and albedo are shown in Figs 1 to 4. For each fraction, heat treatment resulted in a non-linear inactivation of the enzymes. The inactivation

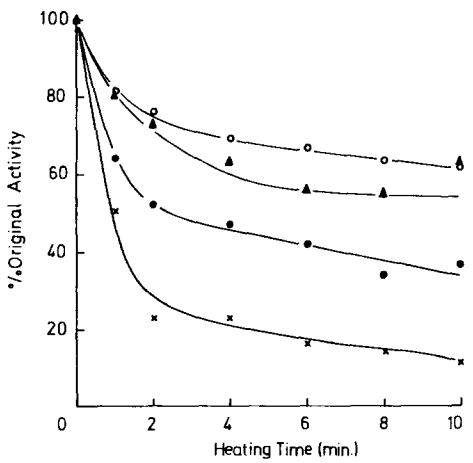

Fig. 1. Heat inactivation of orange juice soluble peroxidase (JSP). ○——○ Heating at 60 °C. ▲——▲ Heating at 65 °C. ●——● Heating at 70 °C. ×——× Heating at 75 °C.

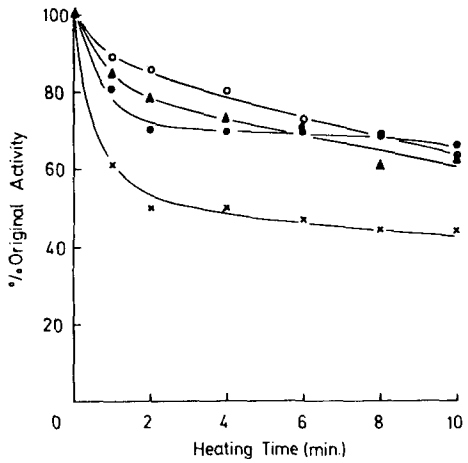

Fig. 2. Heat inactivation of orange juice ionically bound peroxidase (JIP). ○——○ Heating at 60 °C. ▲——▲ Heating at 65 °C. ●——● Heating at 70 °C. ×——× Heating at 75 °C.

curves obtained at each temperature indicate that the heat inactivation process is at least biphasic, and, therefore, that each extract may contain a number of peroxidases which differ in their stability to heat. The ionically bound peroxidases from both juice and albedo fractions appear to be more heat stable than the corresponding soluble peroxidases. This is in contrast to results obtained previously for Brassica peroxidase enzymes

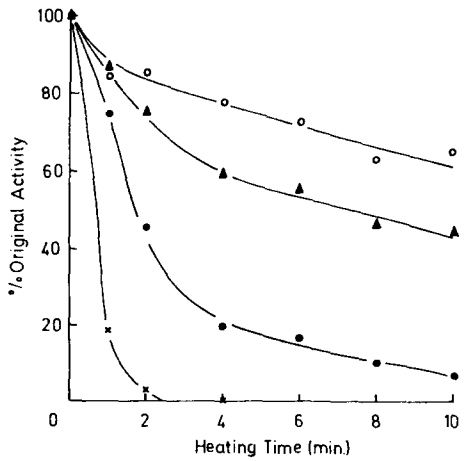

Fig. 3. Heat inactivation of orange albedo soluble peroxidase (ASP). ○——○ Heating at 60 °C. ▲——▲ Heating at 65 °C. ●——● Heating at 70 °C. ×——× Heating at 75 °C.

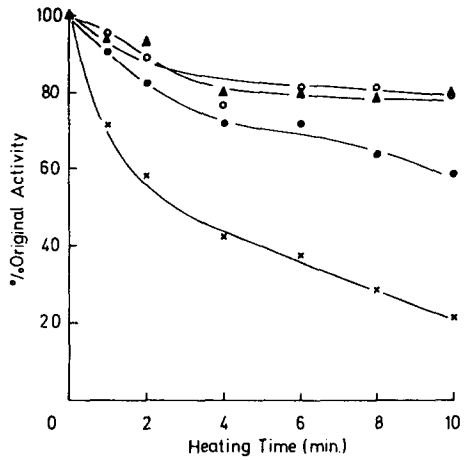

Fig. 4. Heat inactivation of orange albedo ionically bound peroxidase (AIP). ○——○ Heating at 60 °C. ▲——▲ Heating at 65 °C. ○——○ Heating at 70 °C. ×——× Heating at 75 °C.

(McLellan & Robinson, 1981) where the soluble peroxidases were found to be more stable to heating than the bound peroxidases.

Ea values were calculated for the inactivation of peroxidase in each fraction investigated and are shown in Table 2. These values represent the latter phase of heat inactivation, and therefore the more heat stable peroxidases in each fraction. Orange juice soluble and ionically bound peroxidases and albedo ionically bound peroxidases (JSP, JIP and AIP) showed similar Ea values of approximately 80 kJ mol^{-1}, while the value for albedo soluble peroxidase (ASP) was much higher. Thus, the albedo soluble fraction may contain different peroxidase enzymes, responding differently to heat treatment.

The course of regeneration of peroxidase activity at 30 °C against time for the heat treated extracts obtained from orange is shown in Fig. 5. In

TABLE 2
Ea Values for the Inactivation of Peroxidase

Extract	*Ea* ($kJ\,mol^{-1}$)
Juice soluble peroxidase (JSP)	74·5
Juice ionically bound peroxidase (JIP)	87·8
Albedo soluble peroxidase (ASP)	203
Albedo ionically bound peroxidase (AIP)	81·1

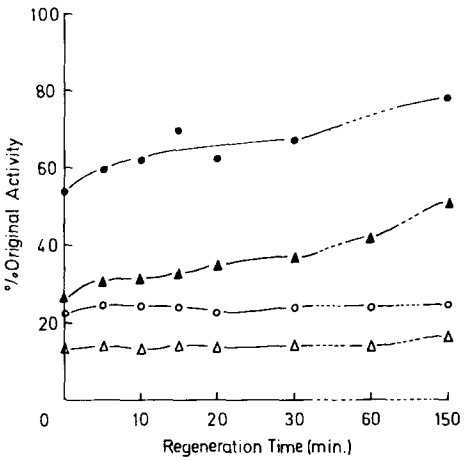

Fig. 5. Regeneration of peroxidases extracted from orange. ○——○ Orange juice soluble peroxidase (JSP). ●——● Orange juice ionically bound peroxidase (JIP). △——△ Orange albedo soluble peroxidase (ASP). ▲——▲ Orange albedo ionically bound peroxidase (AIP).

the case of orange juice soluble extracts, only 2% of the peroxidase activity of the fresh unheated extract was found to regenerate. Orange juice ionically-bound peroxidase, however, showed approximately 25% regeneration. Similarly, the albedo soluble and albedo ionically-bound peroxidases showed approximately 5% and 25% regeneration, respectively. It seems, therefore, that the more stable ionically bound peroxidases are capable of considerably greater regeneration than the soluble enzymes. This level of regeneration may result in the presence of active peroxidase after heat treatment. Commercial heat treatment is generally a high temperature–short time process, which Adams (1978) found to be less effective in the irreversible inactivation of horseradish peroxidase than heating at a lower temperature for a more prolonged period. Active peroxidase could catalyse oxidative reactions, leading to quality deterioration in juice exposed to air.

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