ELSEVIER

Food Chemistry 53 (1995) 137–142

0308-8146/95/$9.50

Heat-inactivation of mango pectinesterase and polygalacturonase

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(Received 24 November 1993; accepted 12 June 1994)

The present work involves isolation of mango pectinesterase (PE) and polygalacturonase (PG), and investigating some of its characteristics, mainly with respect to heat-stability of the enzymes. Within a reaction time of 10 min, mango PE shows its maximal activity at pH 7·5 in a reaction mixture containing 1% citrus pectin and 0·1 or 0·2 M NaCl, at 55°C. This enzyme possesses a Z value of 18·5°C. Mango PG reveals its maximal activity in a reaction mixture containing 0·1% Na-polygalacturonate and 0·1 M NaCl. The reaction mixture was adjusted to pH 4·8 (McIlvaine buffer of 0·1 M) and 30–35°C. Mango PG was less heat-stable than PE, with a Z value of 12·25°C. The stepwise running of the inactivation diagrams of PE and PG emphasise the suggestion of the existence of more than one PE and more than one PG in mango enzyme extract.

INTRODUCTION

Pectinesterases (EC 3.1.1.11) are found in the tissues of all plants and many microorganisms. These enzymes have high specificity in hydrolysing the methyl esters of pectin. Mould pectinesterase (PE) cleaves the ester groups randomly in a manner resembling alkali and acid saponification (Baron *et al.*, 1980), while plant PEs attack the pectin chain from the reducing end or next to free carboxyl groups and then proceed linearly along the molecules having blocks of successive galacturonic acid residues with free carboxyl groups which impart the high sensitivity of pectin to calcium ions (Versteeg, 1979). In general, the optimum pH range for plant PE is 7–8 compared with 4–5 for mould PE (Siliha, 1985).

Native PE in fruits may cause desirable and undesirable effects before, during or after processing of fruit juices. PE is one of the enzymes claimed to be responsible for fruit ripening and softening (Krop & Pilnik, 1974).

Pasteurisation of fruit juices is necessary both to prevent microbiological spoilage and to inactivate the native enzymes, principally PE, PG and polyphenoloxidase. It has previously been determined that the thermal resistance of the common spoilage bacteria and yeasts occurring in citrus juices is less than that of PE (Bisset *et al.*, 1953; Patrick & Hill, 1957). However, it was known over 50 years ago that PE activity in orange juice is quite thermostable, surviving temperatures up to 80°C and requiring substantial periods at more than 90°C for complete inactivation (Joslyn & Sedky, 1940). In an attempt to maximise PE destruction with minimum quality loss, Eagerman and Rouse (1976) precisely determined PE inactivation parameters optimally compatible with pasteurisation. For an F_r = 1·0 min, the recommended processes are T = 194°F with Z = 12·2 for orange juice and T = 186°F with Z = 9·3 for grapefruit juice.

Versteeg (1979) was able to identify 12 forms of PEs in Navel orange. Three forms of these were purified and characterised. The optimum pH was about 7·5 and the enzymes were still active at pH 2·5. The D values and Z values for these three forms were: 0·00037 min and 6·5°C for PE_I; 0·0015 min and 11°C for PE_{II}; and 0·375 min and 6·5°C for PE_{III} (a high molecular weight PE). PE_I and PE_{II} (isoenzymes) and the so-called high molecular weight PE were rapidly inactivated at 70, 60 and 90°C, respectively.

Nath and Ranganna (1980) studied the thermal inactivation of PE in Totapuri mango syrup homogenate $F_{208·8}$ = 1·00 and $D_{208·8}$ = 0·456 (at pH 3·6). The F value was equivalent to 2·19 D. In commercial canning a 3 D process is recommended, which is adequate to inactivate the PE. Siddalingu *et al.* (1985) also studied the thermal process schedules for papaya, guava and mango pulps on the basis of inactivation of PE. They found that the D_{100} values were 0·39, 0·34 and 0·33 and the F_{100} values were 1·03, 0·58 and 0·67 those of PE for papaya, guava and mango pulps, respectively.

Murthy (1981) found that the activity of PE is higher in the breakdown pulp than in healthy pulp, and that peels have higher PE activity than the pulp of mango at all stages of ripening (Ashraf *et al.*, 1981). Roe and Bruemmer (1981) found that PE activity declined during the first stage of ripening of mango fruits, but then levelled off. According to Selvaraj and Kumar (1989), softening enzymes, such as PE, polygalacturonase and cellulase, increased, in general, from harvest maturity to colour break stage of mango fruits.

The polygalacturonases (PGs) belong to the depolymerising enzymes (hydrolases) and split the glycosidic bonds between the units of the chain. They are produced by numerous moulds and bacteria and some yeasts, and are also frequently found in higher plants and some insects. In general, PGs are divided into two groups: exo-PG (EC 3.2.1.67), and endo-PG (Rombouts & Pilnik, 1980; Pilnik & Rombouts, 1981). Endo-PGs cause a rapid decrease in the viscosity of pectin solutions without a marked increase in reducing groups. The best substrate for PG action is polygalacturonic acid, but pectin of different degrees of esterification may also be attacked. Two free carboxyl groups at a certain distance apart are required to form binding sites for the enzyme molecule. In the cell wall of higher plants, PG can attack and solubilise native pectin (protopectin) with a high degree of esterification, depending on the distribution of the carboxyl groups along the molecules, because of the action of native PE. Because of this action, PEs are considered to participate in the softening of fruit tissue during ripening (Bartley *et al.*, 1982).

Several studies on tomato PG have been published (Tucker *et al.*, 1981; Zainon & Brady, 1982; Moshrefi & Luh, 1984). On the other hand, no data are available for mango PG and its heat stability.

The present study was directed towards determination of mango PE and PG and investigation of its characteristics (mainly heat stability).

MATERIALS AND METHODS

This study was carried out using the Egyptian mango variety Zebda.

Identification and characterisation of PE and PG

Enzyme extraction from mango pulp and peel

Based on the type of enzyme to be detected, the mango pulp and peel were subjected to a special treatment as shown in Fig. 1. The detection of pectinesterase (PE) titrimetically needs no further fractionation or purification after homogenisation. A flow sheet, illustrating the sequences of enzyme extraction and separation is given in Fig. 1.

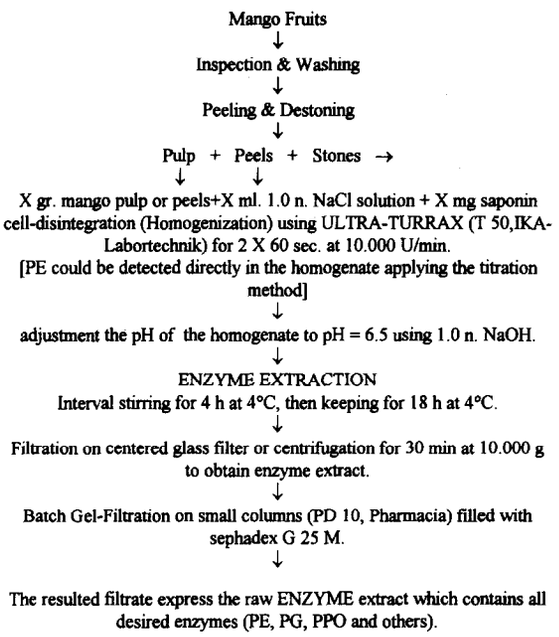

Fig. 1. Extraction of some native enzymes of mango peels or pulp.

Pectinesterase (PE) assay

The method of Rouse and Atkins (1952) was adapted and modified for the determination of mango PE activity. Mango enzyme extract (10 ml) was mixed thoroughly and rapidly for 30 s with 20 ml of 1% pectin (citrus pectin 72% DE) solution in 200 mM NaCl (titrated with 100 mM NaOH to pH 7·5) and the pH rapidly adjusted to 7·5 with 100 mM NaOH. The pH of the reaction mixture was then maintained at 7·5 for 15 min by adding 10 mM NaOH with a burette. The PE activity was expressed as the microequivalents of ester hydrolysed per minute (PE units) per gram of mango at pH 7·5 and 30°C.

Polygalacturonase (PG) assay

Activity of polygalacturonases could be followed by measuring the increase of reducing groups due to the enzyme action on the glycoside bonds. In this study, three methods were tested: the photometric method using dinitro salicylic acid (DNS) according to Miller (1959), copper–arsenomolybdate reagents according to Somogi (1952) and the use of the 2-cyanoacetamide reagent according to Honda *et al.* (1982) on the pure monomers, glucose, galacturonic acid and arabinose. It was found that the use of the 2-cyanoacetamide reagent was more effective for the detection of the traces of PG in fruit extracts, because of the lower limit of end-product detection. The results were calculated as units of PG. One unit of PG is the amount of enzyme which catalyses the release of 1 μmol of reducing groups per minute under suitable conditions using polygalacturonic acid as substrate (Honda *et al.*, 1982). Na-polygalacturonate in a concentration of 0·1% was used as substrate solution.

Effect of some factors on PE and PG activity

The optimum reaction time and enzyme concentration as well as the effects of pH, temperature and NaCl on

the activity of the tested enzyme present in the raw extract, were investigated and optimised. In this respect, the classical biochemical methods of Dixon and Webb (1979) were applied. The buffer systems used were prepared according to the methods of Gomori (1955).

To study the thermostability of the tested enzyme, pulp and peel extracts were distributed in small test-tubes having the same length, diameter and wall thickness. The tubes were incubated in a water bath at various temperatures (45–85°C) and for different periods (0–30 min), and cooled in tap water. The residual activity was measured immediately as described before. The log of the residual activity (%) was calculated and plotted against treatment time using semi-log paper. The decimal reduction times (*D* values; the time in minutes required to inactivate 90% of the enzymes under investigation) were read from the straight lines on the figures at different temperatures and used for drawing thermal destruction curves. The *Z* value is the rise in temperature necessary to observe a ten times faster heat-inactivation, or to reduce the *D* values to 1/10 (90% reduction in *D* values). The warming-up time was eliminated from the *D* values by using the straight part of the lines in the destruction curves.

RESULTS AND DISCUSSION

Distribution of PE and PG in mango pulp and peel

The results indicate that the activity of PE and PG in mango peel is higher than in the pulp, being 39·0 and 0·75 units/100 g peel and 4·5 and 0·55 units/100 g pulp, respectively. The fact that mango peel contains more than eight-fold the PE activity of that in pulp is of importance, especially when mangoes are processed without peeling, and also indicates the importance and the conditions of fruit and pulp blanching 'Thermobreak'. According to Ashraf *et al.* (1981), the peels have higher PE activity than the pulp in all mango varieties tested. The loss of firmness in ripening mango was reported to be correlated with an increase in PE, PG and especially in cellulase activity (Murthy, 1981; Rao & Bruemer, 1981; Selvaraj & Kumar, 1989).

Properties of mango PE

Reaction time

The PE activity course within a period of 15 min at pH 7·5 and 30°C was studied. Due to the continuous decrease of ester degree (DE) of the substrate as well as the accumulation of the methanol, the reaction velocity also decreased. In the first 8 min, the reaction runs as a first-order reaction in which the graphical relationship between reaction velocity and reaction time is linear. Thereafter, the reaction velocity decreases. Versteeg (1979) reported that citrus PE was competitively inhibited by low-esterified pectin and/or short chains of pectic acid. The same interpretation could be concluded for mango PE due to the presence of PG in the same enzyme extract. A reaction time of 10 min was chosen, and the slope of the straight region of the reaction curve was calculated and given as enzyme activity.

pH optimum

Within a reaction time of 10 min and at 30°C, the activity of PE in mango enzyme extract was measured using citrus pectin (72% DE) as substrate at different pH values. PE had its optimum at pH 7·5 and was still active at pH 5·5 (77%). Below pH 5·5 the activity decreased drastically, and no activity could be detected at pH 3 or below. The wide and smooth increased peak of activity from pH 5·5 to 8·0 suggested the presence of more than one type of PE in mango enzyme extract. Citrus PE has a pH optimum of 7·6 (Versteeg, 1979).

NaCl concentration

According to various reports, plant PE can be activated by Na^+ and Ca^{2+} ions. In this experiment PE activities in mango pulp as well as peel extracts were followed for 10 min at different NaCl concentrations in reaction mixtures adjusted to pH 7·5 and 30°C. The maximum PE activity of mango peel extract (100%) was at 0·1 M, and the activity decreased gradually and smoothly up to 0·4 M, which showed at this concentration more than 60% of the activity. For PE activity of mango pulp extract, there were two NaCl optimum concentrations (0·1 and 0·2 M). The wide peak of peel PE and the two peaks of pulp PE pointed to the presence of more than one type of PE in each enzyme extract. This finding is in accordance with the finding of Versteeg (1979), who found more than 10 isoenzymes of PE in Valencia orange. Both enzyme extracts showed no PE activity at 0·45 M NaCl. By carrying out the experiment without NaCl, the pulp enzyme extract showed a PE activity of about 50% whereas peel extract showed 75% of PE activity. In general, 0·1 and 0·2 M were used in the standard reaction mixtures for measuring the activity of PE in peel and pulp enzyme extract, respectively.

Temperature

PE activity was measured at different temperatures under standard conditions of pH, NaCl and reaction time. In the range between 25 and 55°C, the activity of PE was almost linear (first-order reaction) and dropped dramatically thereafter. This phenomenon needs no special interpretation and is in accordance with normal enzyme behaviour and heat-activation of chemical reactions according to the Arrhenius law.

To test PE enzyme stability at various temperatures, enzyme extract was treated for a fixed time of 20 min at various temperatures at pH 3·8 (the natural pH of mango puree) and at pH 7·0. After cooling in ice water, the residual activity was assayed immediately under standard conditions. The experiment was carried out using both mango pulp and peel extracts. The results of both extracts showed the same trend. For both treatments (at pH 3·8 and 7·0), the enzyme was stable up to 30°C and possessed 100% activity. The enzyme extracts

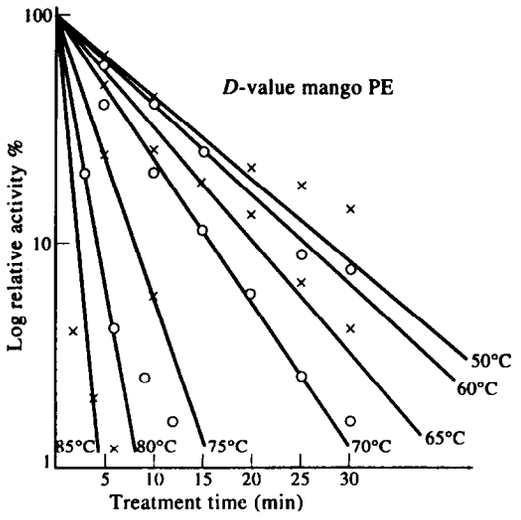

Fig. 2. Calculation of *D*-values for mango PE at different temperatures.

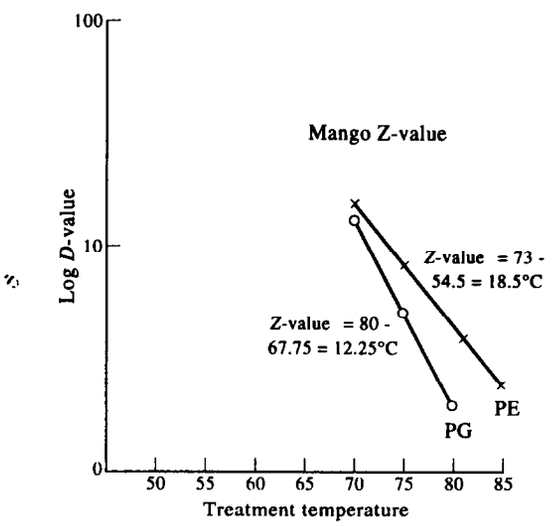

Fig. 3. Thermal destruction curves (TDCs) of mango PE and PG.

treated for 20 min at 40°C showed decreased residual activity either at pH 3·8 or 7·0. The remaining PE activity was 84% and 93%, respectively. This means that the heat-denaturation of the enzymes is still limited. Treatments at temperatures above 40°C up to 65°C, caused a strong denaturation, which resulted in a marked decrease in PE activity. The stepwise inactivation of PE activity may confirm the presence of more than one type of PE in the extract as suggested earlier. In general, 30°C was recommended to estimate PE activity.

Thermostability

To obtain a definition for heat stability, the *D* and *Z* values of the enzymes under investigation were calculated. Enzyme extract was treated as in heat-inactivation experiments, but incubation times at the different temperatures were varied. The log of the residual activity (%) was calculated and plotted against incubation time in destruction curves as shown on Fig. 2.

The *D* values at the different temperatures were read from the figures and used for thermal destruction curves (TDCs). The TDCs for all enzymes under investigation are shown in Fig. 3 (for comparison), and the calculated *D* and *Z* values for all enzymes are listed in Table 1.

Table 1. Calculated *D* and *Z* values of mango PE and PG

	D values (min)	
Temperature (°C)	PE	PG
50	27·5	33·7
60	25	19
65	20	16
70	15·5	13
75	8	5
80	4·5	2
85	2·5	
Z values (°C)	18·5	12·25

The results indicate that the inactivation of the crude PE follows a complicated pattern and no real *D* values could be obtained at temperatures below 70°C. This behaviour may be due to the presence of several types of PE in mango extract with different heat stabilities. In older literature, irregular heat-inactivation patterns of citrus PE were also shown (Bissett *et al.*, 1953; Rouse & Atkins, 1955). In some other works, no irregularities in heat-stability of PE of citrus juice were reported (Eagerman & Rouse, 1976; Nath & Ranganna, 1977).

The calculated *Z* value for PE is rather high (18·5°C) compared with those values obtained for PE of orange, mandarin or grapefruit: 11, 11·4 and 5·2°C, respectively (Eagerman & Rouse, 1976; Nath & Ranganna, 1977; Versteeg, 1979). In more recent work the *Z* values of orange juice pulp PEs were estimated to be 6·5 and 10·8°C for the sensitive and stable fractions (Wicker & Temelli, 1988). Omran *et al.* (1991) reported a *Z* value of 15°C for cucumber PE.

In summary, the calculated *Z* value in this work is almost an average of different high *Z* values of mango PEs. These PEs seem to be heat-resistant enzymes, and their nonlinear inactivation at low temperatures suggests the presence of more than one type of PE with different heat stabilities. According to Versteeg (1979), heat-stability of purified orange PE increased on increasing sugar concentration. A high heat-stability of PE could thus be possible for PE in raw mango juice.

Properties of mango PG

Reaction time

To study the linearity range during PG reaction, the activity of PG was followed for 2 h at pH 5·0 (acetate buffer) and 30°C. The reaction course was linear (first-order reaction) in the first 60 min, then decreased up to 80 min. The reaction velocity in the first 60 min was constant, and decreased thereafter. In the next experiment a reaction time of 30 min was chosen.

NaCl concentration

As with mango PE, activity of mango pulp PG was assayed in the presence of various amounts of NaCl in reaction mixtures (0·05–0·5 M), at pH 5·0 (acetate buffer) and 30°C. The demonstrated NaCl-activity curve indicated two optimum concentrations, one at 0·1 M and the other at 0·35 M. The activity at 0·1 M NaCl was about two-fold of that at 0·35 M NaCl, with a marked decrease in-between and thereafter. These results suggest the presence of at least two enzymes in the raw enzyme extract. A NaCl concentration of 0·1 M was used in the next experiments.

pH optimum

Mango PG activity was studied at different pH values (3·6–6·4) in two buffers: Na acetate and McIlvaine buffer (0·1 M) under standard conditions. The activity in Na acetate buffer showed a stepwise increase with a peak maximum at pH 5·0, then decreased rapidly to reach about 30% at pH 5·4. On the acidic side of the optimum pH, PG activity was about 40–50% (around pH 4, the natural pH of mango pulp). Concerning the activity in McIlvaine buffer, there was a wide peak of activity between pH 4·2 and 5·0, with a maximum activity at pH 4·8. This wide peak is the result of more than one peak (which overlapped to produce a wide one). The activity at pH similar to that of mango pulp (around pH 4) was about 90%. The activity decrease around the maximum pH was rather smooth, and the curve shows, in its general course, a stepwise trend. This establishes the presence of more than one PG type in the raw enzyme extract.

The work in the next experiments was carried out by applying McIlvaine buffer at pH 4·8, 0·1 M.

Temperature

The activity of mango PG was assayed under standard conditions as a function of temperature (20–60°C). The maximum activity of mango PG was at 30–35°C, and dropped rapidly at higher temperatures in a stepwise manner to reach about 15% at 60°C. The stability of mango PG at various temperatures (treatment of enzyme extract for 20 min at various temperatures) was done as described before. The enzyme was rather stable up to 25°C, whereas at 30°C and 35°C, 95% and 90% activity were retained. At higher temperatures, the enzyme stability decreased gradually to about 5% of the residual activity at 60°C. From the above results, 30°C is recommended to assay PG activity within 30 min reaction time and at pH 4·8.

Thermostability

The constants of heat-stability, namely *D* and *Z* values, of mango PG, were calculated and are shown in Fig. 4, from which *D* values at various temperatures were calculated and are shown (with others) in Table 1. The calculated *D* values at 70, 75 and 80°C were used to draw TDCs (Fig. 3) to calculate *Z* values of PG (Table 1).

The irregularity in heat-stability due to the complicated pattern of heat-inactivation was also noted for PG in mango crude enzyme extract, especially below

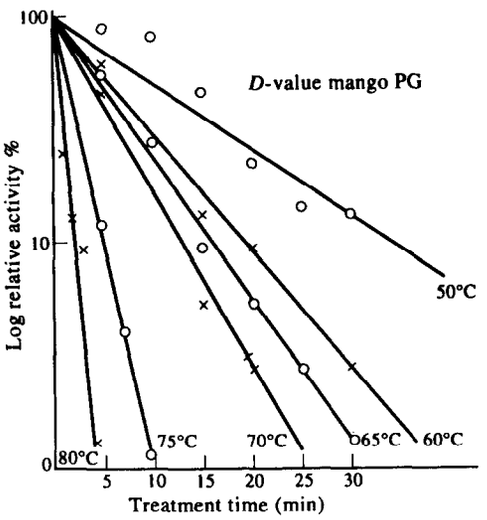

Fig. 4. Calculation of *D* values for mango PG at different temperatures.

70°C. This could be interpreted by the presence of more than one type of PG in the crude extract. The *Z* value calculated for mango PG (12·25°C) was lower than that of PE of the same extract. This means that PG is less heat-stable than PE (18·5°C). This was also reported for the crude enzyme of cucumber (Omran *et al.*, 1991). The heat-stability of PG (similar to PE) may be higher in raw pulp than in crude enzyme extract. In the available literature there are no comparable figures for *D* and *Z* values for mango PG.

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