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Extraction and characterisation of pectin methylesterase from black carrot (*Daucus carota* L.)

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ABSTRACT

This study was carried out to determine some of the biochemical properties of pectin methylesterase (PME) from black carrot. The enzyme showed very high activity in a broad pH range of 6.5–8.5, with the optimum pH occurring at 7.5. The optimum temperature for maximal PME activity was found to be 55 °C. NaCl enhanced PME activity, particularly at 0.2 M. K_m and V_{max} values for black carrot PME using apple pectin as substrate were found to be 2.14 mg/ml ($r^2 = 0.988$) and 3.75 units/ml, respectively. The enzyme was stable between the temperatures of 30–50 °C/5 min whereas it lost nearly all of its activity at 70 °C/5 min. E_a and Z values were found to be 196.8 kJmol^{-1} ($r^2 = 0.996$) and 2.16 °C ($r^2 = 0.995$), respectively.

1. Introduction

Pectin methylesterases (PME, EC 3.1.1.11) have been detected in plants as well as in pathogenic fungi and bacteria. Multiple forms of PME (basic, neutral, and acidic isoforms) can be present within each species. These isoforms have different biochemical properties. In general, the plant and bacterial PMEs have pH optima between 6 and 8, whereas some fungal PMEs have pH optima between pH 4 and 6. Plant PMEs require NaCl for the optimal catalysis. PMEs are cell wall-bound enzymes and catalyse the hydrolytic cleavage of the methyl ester moieties on pectin molecules, resulting in the release of methanol and partially de-esterified pectin (Sila et al., 2007). Because structure is one of the most important characteristics, it is essential to study cell wall polysaccharides and cell-wall degrading enzymes for a better understanding of the system and to achieve desired end-product quality. Pectic polysaccharides represent a large portion of the primary cell walls and middle lamella. They contribute to the mechanical strength of the cell wall and to the adhesion between cells; for that reason, pectin is intimately connected to fruit and vegetable firmness (Nunes et al., 2006).

The structure of the pectic polysaccharides can be altered during fruit ripening or processing caused by the activity of specific cell wall-bound enzymes, mainly PME and polygalacturonase (PG). PME catalyses the de-esterification of pectic polysaccharides from cell walls, which can be further hydrolysed by PG, resulting in a decrease of the degree of polymerisation of the pectin chains and a loss of firmness of the tissue. On the other hand, de-esterified pectic polysaccharide chains may form crosslinks with bivalent ions to form pectate gels, and are less sensitive to β-eliminative breakdown during thermal treatments. These features increase the firmness of the cell wall. Therefore, PME can have a beneficial effect on the texture of fruits and vegetables (Nunes et al., 2006).

It was demonstrated that stimulation of PME activity before cooking in carrots enhanced the texture of the final product significantly. The demand for carrot based juices and concentrates is increasing and the extraction yields have been effectively improved using PME together with other pectinases. PME is used widely in the processing of clarified juices. However, it has deleterious effects in cloudy juices and concentrates. The control of PME activity has been a common subject of study because of the implications in the modification of the texture of fruit and vegetables and as a destabilising agent of pectin materials in fruit juices and concentrates (Sila et al., 2007).

The so-called black or purple carrot (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) originated from Middle Asia, where it has been known for ~3000 years. It was not cultivated in Europe until the 12th century and is considered to be the archetype of all modern orange carrots, which were bred by Dutch growers around 1750 (Schwarz, Wray, & Winterhalter, 2004). In Turkey, a fermented drink called 'Shalgam' is produced from black carrots. Black carrots are also used for production of extracts rich in acylated anthocyanins which serve as a major source of natural food colours for the food colourant industry. Recently it has been demonstrated that anthocyanin-rich extract from black carrot inhibited proliferation of human cancer cells (HT-29 colorectal adenocarcinoma and

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HL-60 promyelocytic leukaemia) in a dose-dependent manner (Netzel et al., 2007).

There has been extensive research on PME from different sources, e.g. orange carrots (Anthon & Barrett, 2006; Balogh, Smout, Ly-Nguyen, Van Loey, & Hendrickx, 2004; Ly-Nguyen et al., 2002a; Vora, Kyle, & Small, 1999), cucumber (Yemenicioglu & Cemeroglu, 1999), green beans (Laats, Grosdenis, Recourt, Voragen, & Wichers, 1997), orange juice (Lee, Lin, Chang, Chen, & Wu, 2003), pepper (Castro, Van Loey, Saraiva, Smout, & Hendrickx, 2006). However, to the best of our knowledge, no work has been done on PME from black carrot. The present work was undertaken to study the characteristics of PME from black carrot in terms of pH and temperature optima, thermal stability and inactivation, kinetic parameters and effect of salt.

2. Materials and methods

2.1. Materials

The black carrots used in this study were obtained from Konya-Eregli region of Turkey and kept at 2 °C until used.

Apple pectin (70–75% esterification degree), trisma base, and dialysis bags (cellulose membrane: 76 mm × 49 mm) were purchased from Sigma (St.louis, USA) and sodium hydroxide Titrisol, ethanol (99%), ammonium sulphate, hydrochloric acid, sodium disulphite, polyvinylpolypyrrolidone (PVPP) and sodium chloride were purchased from Merck (Darmstadt, Germany). All the reagents were analytical grade.

2.2. Extraction of PME

PME is ionically bound to cell wall and therefore a buffer with a high ionic strength (Tris, NaCl) is needed to extract it from cell walls. To prevent PME losses due to high phenolic content of black carrots, they were washed with $Na_2O_5S_2$ and PVPP before and during extraction (Nunes et al., 2006).

The extraction of carrot PME was performed according to the methods of Denes, Baron, and Drilleau (2000) and Ly-Nguyen et al. (2002a) with modifications. All extraction steps were performed at 4 °C to prevent enzyme inactivation. Carrots in batches of 200 g were chopped and then homogenised in 300 ml of cold distilled water containing 500 mg/l sodium disulphite for two minutes at maximum speed by using a Waring blender (Model HGB2WTS3, Torrington, Connecticut, USA). The homogenate was filtered through cheesecloth. The pellet was then washed with 1200 ml cold distilled water to remove other organic and colour compounds. The washing and filtering were performed three times. The pellet was mixed with 320 ml of cold distilled water containing 500 mg/l sodium disulphite and then centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was discarded. Mixing and centrifuging were performed three times. The last pellet was resuspended in 160 ml of cold buffer solution of 20 mM Tris–HCl (pH 7.5) buffer containing 1 M NaCl and 500 mg/l $Na_2O_5S_2$, followed by centrifugation (10000*g* for 30 min at 4 °C). 1% (w/v) PVPP (polyvinylpolypyrrolidone) was added to the supernatant and magnetically stirred for 30 min at 4 °C, followed by centrifugation at 5000*g* for 30 min at 4 °C. This procedure was repeated until a clear, colourless supernatant was obtained. Altogether, a total of 1 kg of black carrots was used in the extraction of PME.

All the supernatants obtained at each extraction step were combined and then subjected to 80% ammonium sulphate precipitation. The precipitate containing PME was collected by centrifugation at 10000*g* for 30 min at 4 °C and dissolved in 3 ml 10 mM Tris–HCl (pH 7.5). The extract was then dialysed overnight in Tris–buffer (pH 7.5) at 4 °C. The extract was used as the PME source in the following experiments.

2.3. Assay of PME activity

PME activity was measured titrimetrically by determining free carboxyl groups formed as a result of enzyme action on pectin. The reaction mixture was composed of 20 ml of 0.5% apple pectin solution containing 0.1 mol/l NaCl and 0.5 ml of PME extract. The reaction was carried out at 30 °C in a water-jacketed reaction beaker. The volume of NaOH required to maintain the pH of the reaction mixture at 7.5 for 10 min was measured. One unit of PME was defined as the amount of enzyme that released 1 μmol of carboxyl groups/min, under the aforementioned assay conditions. PME activity was calculated using the following formula:

PME (units/ml) = (ml of NaOH)(molarity of NaOH)(1000)/(time)(ml of enzyme).

2.4. Kinetic parameters

In order to determine Michaelis constant (K_m) and maximum velocity (V_{max}), PME activities were measured using apple pectin as substrate at various concentrations (0.125–4 g/l). K_m and V_{max} values of the enzyme were calculated from a plot of 1/V vs. 1/S by the method of Lineweaver and Burk.

2.5. pH Optima

The pH dependence of PME activity was assayed in a pH range of 6.0–9.0, using the standard reaction mixture. Blanks without PME extract were made for each determination, and the amount of acid produced due to the spontaneous pectin demethylation was subtracted. PME activity was calculated in the form of percent residual activity at the optimum pH. The optimum pH obtained for this enzyme was used in all other studies.

2.6. Temperature optima

Temperature optima of PME activity were tested under standard assay conditions at temperatures ranging from 20 °C to 70 °C. The temperature was controlled by means of a circulating water bath. Blanks without PME sample were made for each determination, and the amount of acid produced due to the spontaneous pectin demethylation was subtracted. PME activity was calculated in the form of percent residual activity at the optimum temperature.

2.7. Thermal stability

Thermal stability of black carrot PME was determined at temperatures ranging from 30 °C to 70 °C, using screw-cap tubes. The screw-cap tubes were pre-heated to the selected temperature to prevent temperature lag before addition of enzyme solution. The enzyme samples were removed from water bath after 5 min and were immediately transferred to an ice bath to stop thermal inactivation. The residual activity was determined within 60 min as described above.

2.8. Thermal inactivation kinetics

The enzyme samples were incubated for 2.5, 5.0 and 7.5 min at 55 and 60 °C and 7.5, 10.0, 12.5 min at 65 °C in screw-cap tubes. The screw-cap tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of enzyme solution. The enzyme samples were removed from the water bath after preset times and were immediately transferred to an ice bath to stop

thermal inactivation. After the enzyme sample was cooled in the ice bath, the residual activity (A) was determined within 60 min as described previously. A non-heated enzyme sample was used as the blank (A_o). The percentage residual activity was calculated by comparison with the unheated sample. First order inactivation constant (k_D) was calculated from the slope of the natural logarithm (ln) of A/A_o vs. time graph. Half-life of the enzyme ($t_{1/2}$) was calculated by using the following equation: $t_{1/2} = 0.693/k_D$.

Decimal reduction time (D value) was estimated from the relationship between k_D and D value: $D = \ln(10)/k_D$. The Z value, which is the temperature increase required for a one-log10 reduction (90% decrease) in D value, was determined from a plot of $\log_{10}^{D}$ versus temperature. The slope of the graph is equal to $1/Z$ value. The energy of activation of denaturation (E_a) was calculated by multiplying the slope of Arrhenius plot (i.e. natural logarithm of k_D values vs. reciprocal of absolute temperatures ($1/T$)) with universal gas constant, R (kJ mol^{-1} K^{-1}) (Marangoni, 2003).

2.9. Effect of NaCl

Effect of salt on PME activity was determined using 0.5% apple pectin solution containing NaCl in a range of 0–0.5 M.

3. Results and discussion

3.1. Kinetic parameters

K_m and V_{max} values for black carrot PME using apple pectin as substrate were found to be 2.14 mg/ml ($r^2 = 0.988$) and 3.75 units/ml, respectively. K_m value is a measure of affinity of the enzyme for the substrate, with smaller values representing greater affinity. Some of the reported K_m values of PME, isolated from different sources, include 0.031 mg/ml for carrot (*Daucus carota* L.) (Alonso, Canet, Howell, & Alique, 2003), 0.154 mg/ml for carrot (*Daucus carota* var. Nerac) (Sila et al., 2007), 0.098 mg/ml for apple (*Golden* sp.) (Denes et al., 2000), 0.152 mg/ml for banana (Ly-Nguyen et al., 2002b), and 0.274 mg/ml for grapefruit (Guivarc'h, Segiova, Van Loey, & Hendrickx, 2005). As can be seen, K_m value changes depending on the enzyme source. The K_m value obtained in this study was higher than those reported for carrot PME. However, it is difficult to compare the K_m values because the K_m values are dependent on temperature, salt concentration, pectin source, and pH of the reaction medium (Goldberg, Pierron, Durand, & Mutaftschiev, 1992).

3.2. pH Optima

Enzyme activity is strongly affected by the pH of the medium because amino acid side chains in the catalytic centre may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionisation, and ionised side chains may play an essential role in maintaining protein structure (Whitaker, 1996). PME activity as a function of pH was determined in a pH range of 6.0–9.0, and the results are depicted in Fig. 1. As can be seen from the graph, as the pH increased from 6.0 to 7.5, the enzyme activity increased, with the maximal activity occurring at pH 7.5. The enzyme had very high activity in a broad pH range of 6.5–8.5. A sharp drop in enzyme activity was observed after pH 8.5.

Some of the reported optimum pH values for PME from different sources are 7.4 for carrot (*Daucus carota* L.) (Alonso et al., 2003; Ly-Nguyen et al., 2002a), 8.0 for carrot (*Daucus carota* var. Nerac) (Sila et al., 2007), 7.5 for plum (*Prunus domestica*) (Nunes et al., 2006), and 8.0 for peach (Javeri & Wicker, 1991). The pH optimum of 7.5 found in this study was in accord with these values.

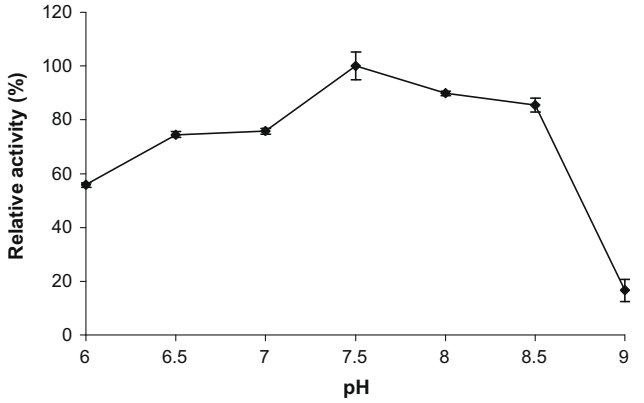

Fig. 1. Activity of black carrot PME extract as a function of pH. Each data point is the mean of minimum of two determinations. The vertical bars represent standard deviations.

3.3. Temperature optima

Temperature affects both the velocity of enzymatic reactions and stability of the enzyme. Temperature also effects the equilibria of all association/dissociation reactions, solubility of substrates and ionisation of prototropic groups in the catalytic centre of the enzyme and enzyme-substrate complex (Whitaker, 1996). Effect of temperature on PME activity was investigated in the range 20–70 °C and the results are depicted in Fig. 2. As the temperature increased from 20 °C to 55 °C enzyme activity increased. The optimum temperature for maximal PME activity was found to be 55 °C, after which the enzyme activity dropped. The enzyme retained more than 80% activity between 50 and 60 °C.

Reported temperature optima for carrot PME are 49 °C (Alonso et al., 2003) and 50 °C (Lee, Lee, Lee, Choe, & Park, 2001), which are similar to the one found in this study. Some of the reported temperature optima of PME from other sources include 63 °C for apple (Denes et al., 2000) and banana (Ly-Nguyen et al., 2002b), 65 °C for plum (*Prunus domestica*) (Nunes et al., 2006), and 75–80 °C for guava (*Psidium* guajava L.) (Leite, Tadiotti, Baldochi, & Oliveira, 2006).

3.4. Thermal stability

The heat stability of carrot PME was studied by incubating the enzyme in preheated glass tubes for 5 min in the range 30–70 °C. As can be seen from Fig. 3, the enzyme was stable between the temperatures of 30–50 °C. A critical temperature for the

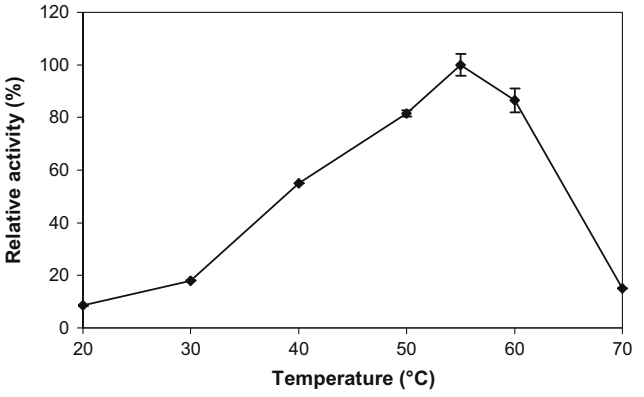

Fig. 2. Activity of black carrot PME extract as a function of temperature. Each data point is the mean of minimum of two determinations. The vertical bars represent standard deviations.

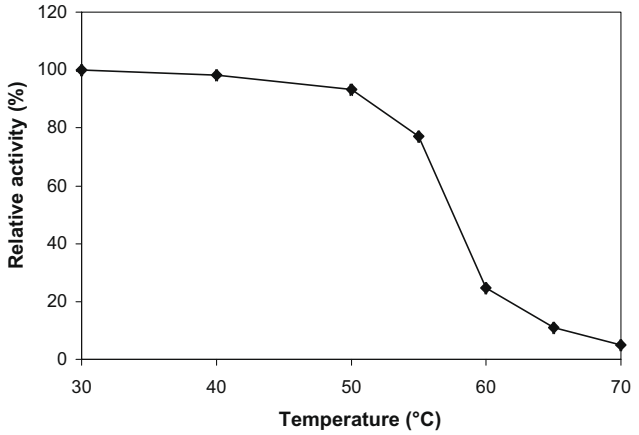

Fig. 3. Thermal stability of black carrot PME extract. Each data point is the mean of minimum of two determinations. The vertical bars represent standard deviations (5 min incubation at indicated temperature).

Table 1
Thermal inactivation parameters of black carrot PME.

Temperature (°C)	k_D (min^{-1})	r^2	$t_{1/2}$ (min)	D (min)
55	0.040	1.000	17.4	57.7
60	0.133	0.989	5.2	17.3
65	0.337	0.944	2.1	6.8

inactivation of black carrot PME was 55 °C because above this temperature the inactivation rate increased greatly. The enzyme lost nearly all of its activity at 70 °C, which indicated that carrot PME was heat sensitive. Alonso et al. (2003) reported similar results for carrot PME. They found that the enzyme was stable up to 40 °C/5 min, with a linear decrease in activity between 40 and 70 °C. In a study carried out by Nunes et al. (2006) on plum PME, it was found that the enzyme retained minimally 90% of its initial activity after 5 min of heat treatment below 50 °C, after which the enzyme activity started to decline gradually. Sila et al. (2007) reported that PME purified from carrot was highly sensitive to heat at temperatures above 50 °C/15 min.

3.5. Thermal inactivation kinetics

The thermal inactivation parameters of black carrot PME are presented in Table 1. The first order inactivation constants (k_D) increased with increasing temperature, indicating that the enzyme was less thermostable at higher temperatures. The half-life ($t_{1/2}$) is another important parameter used in the characterisation of enzyme stability. Increasing the temperature from 55 °C to 65 °C resulted in a decrease in $t_{1/2}$ values (Table 1). Espachs-Barroso, Van Loey, Hendrickx, and Martin-Belloso (2006) who investigated the thermal inactivation of carrot PME reported k_D values of 0.0447, 0.4500 and 1.2200 min at 54, 60 and 63 °C, respectively. They also reported that the thermal resistance of plant PME largely depended on its origin and carrot PME was the least stable among the PMEs investigated.

The decimal reduction time (D value) is the time, at a given temperature and pressure, needed for 90% reduction of the initial activity. D values obtained in this study ranged between 57.7 and 6.8 min at the temperatures studied (Table 1). Denes et al. (2000) reported D values of 2.6 min at 55 °C and 0.7 min at 60 °C for apple PME, which are different than those obtained in this study.

The temperature dependence of the decimal reduction time is characterised by Z value, which is the temperature increase needed for a one $\log_{10}$ reduction (90% decrease) in the D value. The Z value

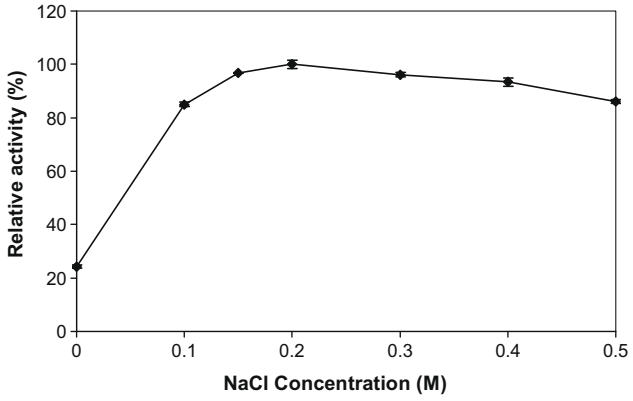

Fig. 4. Effect of NaCl on black carrot PME activity. Each data point is the mean of minimum of two determinations. The vertical bars represent standard deviations.

obtained in this study was 2.16 °C (r^2 = 0.995). Some of the reported Z values include 9.2 °C for apple PME (Denes et al., 2000), 4.71 °C for acerola PME (De Assis, Lima, & De Faria Oliveira, 2000), which are higher than that obtained in this study. E_a value for thermal inactivation of black carrot PME was 196.8 kJ mol^{-1} (r^2 = 0.996). Espachs-Barroso et al. (2006) reported Ea values of 336, 369 and 336 kJ mol^{-1} for banana, tomato and carrot PME, respectively. Anthon and Barrett (2002) reported Ea values of 510 kJ mol^{-1} for labile carrot PME and 635 kJ mol^{-1} for resistant carrot PME.

3.6. Effect of NaCl

The black carrot PME activity was dependent on NaCl concentration as seen in Fig. 4. As the NaCl concentration in the assay mixture increased the PME activity increased gradually and reached the maximum level at 0.2 M. On further increase, the activity declined slightly. Similar results were reported by Leite et al. (2006) for PME from guava. Stimulatory effect of Na^+ ions is attributed to the interactions of Na^+ ions with the substrate (Mc Neil, Davill, Fry, & Albersheim, 1984). Furthermore, it is believed that Na^+ ions bind to the enzyme, inducing conformational changes, favouring reaction of the enzyme with its substrate (Nari, Noat, & Richard, 1991).

4. Conclusions

To the best of our knowledge, this is the first report of the characterisation of PME from black carrot. The enzyme had a high activity in a broad pH range of 6.5–8.5, with the maximal activity occurring at pH 7.5. The enzyme was stable between the temperatures of 30–50 °C/5 min and lost nearly all of its activity at 70 °C, indicating that the PME from black carrot was heat sensitive which was also confirmed by a low Z value obtained in this study. The black carrot PME activity was dependent on NaCl concentration in the assay mixture. The PME from black carrot exhibited similar biochemical properties to that from orange carrots in terms of pH and temperature optima and thermal stability.

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