International Food Research Journal 23(1): 161-166 (2016)
Journal homepage: *http://www.ifrj.upm.edu.my*

Purification and characterization of pectin methylesterase from kiwifruit (*Actinidia deliciosa* var. Hayward)

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Article history

Received: 16 December 2014
Received in revised form: 7 May 2015
Accepted: 9 July 2015

Keywords

Fruit ripening
Pectin methylesterase
Purification
Kiwifruit

Abstract

Pectin methylesterase (PME; EC 3.1.1.11) is widely distributed in plants. PME involved in de-esterification of pectin and have applicability in food, textiles, wines, pulp, and paper industries. In the present study, PME was purified from ripe kiwi fruit (cv. Hayward) using conventional techniques of ammonium sulphate fractionation (30-80% saturation), gel fitration through Sephadex G-75 column chromatography and ion exchange chromatography on DEAE-shephadex. After ammonium sulphate precipitation and dialysis, specific activity of the enzyme was found to be 6.34 units mg^{-1} protein and after chromatoghraphy techniques the specific activity of the enzyme was found to be 64.11 units mg^{-1} protein. Enzyme was purified about 20.75 fold and 37.90 recovery. During optimization of enzyme extraction, the enzyme showed maximum activity at pH 7.5. Stability was found at pH range 7-8, with optimum temperature 30°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) yielded a single major band with molecular weight of 45 kDa indiacting that enzyme was a monomer. The enzyme showed a typical hyperbolic response with increasing concentrations of substrate revealing that it followed Michaelis-Menten kinetics. From the double reciprocal plot, Km value for the PME was found, that is 0.164 mg ml^{-1}. The results obtained in the present study would indicate that Km value of kiwifruit PME was found comparatively lower then other sources. This indicates that ripen kiwifruit PME had higher affinity for the substrate then others.

Introduction

Pectin methylesterase (pectinesterase, PME, EC 3.1.1.11) is widely distributed in plants and microorganisms. PME plays an important role in determining the extent to which pectin is accessible to degradation by polygalacturonase (PG). In plants, PME is bound to the cell wall by electrostatic interaction. It catalyzes the de-esterification of methyl esters of polygalacturonic acid polymers to form pectinic acids/pectic acids and methanol. Pectin methylesterases (PMEs) hydrolyze the methylester groups that are found on the homogalacturonan (HG) chains of pectic polysaccharides in the plant cell wall (Mercadante *et al.*, 2014). Detrimental effects of PME activity on cloud stability of juices and nectars have been reported in detail (Kertesz, 1951; Alonso *et al*., 1997). In contrast, beneficial effects of PME, includes the enhancement of firmness of processed fruit and vegetable products (Powell *et al*., 1982; Ben-Shalom *et al*., 1985) and effective increment of extracting yield of juices by conventional approaches (Krop and Pilnik, 1974). In fruit juice cloud particles impart the characteristic color and flavor. Pectin, a major component of fruit juice cloud, is thought to play an important role in juice destabilization: in the presence of the active enzyme pectin methylesterase (PME), pectin forms calcium pectate complexes and causes the precipitation of cloud particles. In the clarification of fruit juices, the PME enzyme may also be used alone. However, such a treatment needs the addition of $CaCl_2$ to fruit juice. This removes pectin in fruit juice as insoluble calcium pectates and causes the clarification (Massiot *et al*., 1997; Alkorta *et al*., 1998; Wicker *et al*., 2002).

Mature kiwifruit show a rapid drop in firmness after harvest until the fruit reach a firmness of »25 N (2.5 kgf). In the early stages of kiwifruit softening in response to ethylene, the most obvious changes in the cell wall components are swelling of the cell wall and loss of galactose from pectic polymers (Redgwell *et al*., 1990). De-esterification of pectins and some breakdown of middle lamella pectic polymers may also be occurring. PME has been extracted and purified from different plant sources and characterized in terms of biochemical properties and thermal stability. In this paper, PME was extracted from kiwifruit and purified using chromatography techniques. Purified kiwifruit PME was characterized in terms of biochemical properties. PME has been purified

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and characterized from a number of fruits including tomato, peach, lemon, sweet cherry, guava and marsh white grape fruit. However, not much information is available on the characteristics of PME from kiwi fruit.

Materials and Methods

Kiwi fruit samples of cv. Hayward were procured from the orchards of NBPGR regional station, Bhawali at three stages of maturity viz. immature Green (IG) in the month of October, mature green (MG) in the month of November and ripe (R) in the month of December. Bhowali is situated at 290 20' N latitudes 790 30' E longitudes at an altitude of 1600 msl in cold humid, sub-temperate climate with an average annual rainfall of 1200-1600 mm and temperature ranges from -0.08 to 33^{0}C (mercury drops even upto - 7^{0}C during December to February). The harvested fruits free of visible defects were washed thoroughly in sterilized distilled water and air dried.

Chemicals

All the chemicals and biochemical used during the present course of investigations were of analytical grade and obtained from Sigma Chemical Company, St. Louis, M.D., USA, E. Merck, Bombay, Himedia Laboratories Limited, Bombay and Sisco Research Laboratories Pvt. Ltd., Bombay.

Pectin methyl esterase extraction and assay

Pectin methylesterase was extracted and assayed by the method of Hagerman and Austin (1986). The collected samples (IG, MG, R) were analyzed for the analysis of pectinmethyleesterase (PME) activity and found that PME activity increased linearly throughout ripening, attaining their maximum values at ripe stage. So the ripe stage of kiwi was selected for the further extraction and purification of the PME enzyme. In brief, fruit tissue (25 g) was homogenized with 50 ml of chilled 0.1 M Tris-HCl buffer (pH 7.5), containing 10 per cent NaCl. Homogenate was extracted in ice for 1 h with slow and constant stirring before centrifugation at 10,000x g for 30min. The supernatant represented the enzyme extract. The reaction mixture contained 100 µl of enzyme extract, 2.5 ml of 0.5 per cent (w/v) apple pectin in buffer (2 mM Tris-HCl, pH 7.5) and 0.4 ml of 0.01 per cent (w/v) bromothymol blue in the same buffer. The change in absorbance at 620 nm for 30 min was converted to galacturonic acid from the standard curve (50 to 500 µg) prepared under the same assay conditions. Enzyme activity was expressed as mg galacturonic acid released for 30 min g^{-1} f. wt. One enzyme unit was expressed as the amount of enzyme required to release 1 mg of galacturonic acid/30min.

Enzyme purification

The crude enzyme preparation was purified using following techniques. All steps of purification were carried out at 4ºC.

$(NH_4)_2SO_4$ fractionation

The crude enzyme preparation (50 ml) was subjected to 30-80 percent $(NH_4)_2SO_4$ saturation (Green and Hughes, 1955). The precipitates were centrifuged at 10,000 x g for 20 min, dissolved in 0.1 M Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer for 24 h with repeated changes of the buffer.

Ion-exchange chromatography

Five grams of DEAE- Sephadex G-25 powder was suspended in 200 ml of double distilled water and kept overnight at room temperature. Next day one time washed with double distilled water. The anion exchanger was then transferred in 0.5N NaOH for half an hour so as to make it alkaline afterwards washed with distilled water till pH. 7.0, then It was then suspended in 200 ml of 0.5 N HCl for half an hour to make it acidic, immediately filtered through Whatman No. 1 filter paper and washed free of acid with distilled water till pH 7.0 was attained. This preparation was packed in a glass column. After complete sedimentation of the gel, the column with effective bed length was equilibrated with 0.1 M Tris –HCl buffer (pH 7.5) The enzyme preparation obtained after $(NH_4)_2SO_4$ fractionation was concentrated, loaded onto the column and eluted first with 0.1 M Tris –HCl buffer (pH 7.5) and then with a linear gradient of 0.1 – 0.4 M KCl in the same buffer at a flow rate of 0.5 ml/min. The fractions of 2 ml each were collected and analyzed for protein (280 nm) and enzyme activity. The fractions with high enzyme activity were pooled together, concentrated against sucrose and subjected to gel filtration chromatography.

Sephadex G-75 column chromatography

Five grams of Sephadex G-75 was suspended in 200 ml of distilled water and kept at room temperature for 8h with intermittent shaking and allowed it to swell. The suspension was then washed several times with 0.1 M Tris-HCl buffer (pH 7.5) degassed and packed in a glass column. The column was equilibrated with 0.1 M Tris-HCl (pH 7.5). After passing two bed volumes of same buffer, The

Table 1. Summary of purification of PME from kiwi *(Actinidia deliciosa)* fruits var. Hayward

S. No.	Purification step	Volume (ml)	Total protein (mg)	Total activity	Sp. activity (units mg^{-1} protein)	Fold purification	Yield (%)
1.	Crude extract	50	22.56	69.78	3.09	1.00	100
2.	$(NH_4)_2 SO_4$ fraction (30-80%)	5	8.23	52.19	6.34	2.05	74.79
3.	DEAE- Sephadex A-25	5	1.40	45.32	32.37	10.48	65.56
4.	Sephadex G-75	2.5	0.275	17.63	64.11	20.75	37.90

active fractions collected after DEAE Sephadex column were pooled, concentrated, loaded on to the gel filtration column and eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 2 ml each were collected and monitored on UV-visible spectrophotometer at 280 nm for protein content and were also analyzed for enzyme activity. The fractions with enzyme activity were pooled together stored at 4ºC and further used for investigating various characteristics of the enzyme.

Protein estimation

Protein content in crude extract and the enzyme preparations at various stages of purification was quantified by the method of Lowry *et al*. (1951).

Polyacrylamide gel electrophoresis (PAGE)

To determine the molecular weight and subunit composition of the enzyme, denaturating SDS-PAGE was performed by using method of Laemmili (1970).

Results and Discussion

Purification of pectin methylesterase (PME)

Enzyme from the crude extract (total activity 69.78 units and specific activity of 3.09) was precipitated between 30 to 80 per cent concentration of $(NH_4)_2SO_4$ at 4ºC with overnight precipitation. The precipitates obtained were centrifuged at 12000×g for 30 minutes, dissolved in 0.1 M Tris HCl buffer (pH 7.5) and dialyzed against the same buffer for 24 hrs. The dialysate obtained was concentrated using sucrose and a total volume of concentrated was made upto 5 ml. Concentrated dialysate was found to contain enzyme with specific activity of 6.34 units mg^{-1} protein. This step resulted in about 2.05 fold purification with around 74.79 per cent recovery. The concentrated enzyme (5 ml) was then loaded onto a column of DEAE–Sephadex A-25 (sigma) which had been pre equilibrated with 0.1 M Tris-HCl buffer (pH. 7.5) and eluted step wise with 0-0.4 M concentration of KCl in Tris-HCl buffer applied after 25 ml of eluent. Fractions of 2 ml each were collected and tested for protein and enzyme activity. The enzyme was eluted when 0.2 M KCl concentration was applied as a single peak between fractions 10 to 20 with 10.48 fold purification and 65.56 per cent recovery. This clearly demonstrates that the enzyme protein carried a net positive charge under the experimental conditions. The kiwi PME resembles that of banana PME (Brady, 1976) in this respect. The enzymatically active fractions were pooled, concentrated to a minimum volume (5.0 ml) using sucrose and a volume of 2.5 ml further loaded onto Sephadex G-75 column, previously equilibrated with 0.1 M Tris HCl buffer (pH 7.5). Fractions of 2 ml each were collected and tested for protein and enzyme activity. The enzymatically active fractions were pooled and concentrated using sucrose upto 2.5 ml. The final enzyme preparation exhibited 20.75 fold purification with specific activity of 64.11 units mg^{-1} protein and with 37.90 per cent recovery (Table 1). PME has been purified from different plant sources including germinating Vigna sinensis seeds with 28 per cent recovery, 64 fold purification and specific activity of 390 (Nighojkar *et al*., 1994) and from lemon fruits with 10.8 per cent yield and specific activity of 522.1 (MacDonald *et al*., 1993) by following the conventional techniques of enzyme purification. Sood and Mathur (2014) purified and characterized of pectin methyl esterase from apple pomace. In a recent study the effect of pectin methylesterase on carrot *(Daucus carota)* juice cloud stability was studied and found that PME addition in juice resulted in clarification; higher amounts of enzyme had a modest effect in causing more rapid clarification, due to a faster increase in particle size (Schultz *et al.*, 2014).

A thermally tolerant form of PME has been purified from a commercially available Valencia fresh orange juice by gel filtration, heparin and concanavalin A chromatography (Cameron and Grohmann, 1996). Seymour *et al.* (1991) purified a thermolabile and a thermostable PME from Marsh white grape fruit pulp by ion exchange and gel filtration chromatography with 124 and 309 fold purifications, respectively. Brady (1976) purified two isoform of PME from banana after isoelectric focusing one at pH 8.8 to 8.9 and the other at pH 9.3 to 9.4 with specific activity of 362 and purification factor of 172 and with specific activity of 324 and purification factor of 154, respectively. Similarly, PME from sweet cherry (Alonso *et al*., 1996), persimmon (Alonso *et al*., 1997), mung bean hypocotyl cell walls (Bordenave

and Goldberg, 1993) and ripe peach fruit (Glover and Brady, 1994) has been reported to be separated in four, two, four and three isoforms respectively.

Characterization of partially purified enzyme

The final enzyme preparation after gel filtration chromatography was used for investigating various characteristics of enzyme protein.

Molecular weight determination

The molecular weight of purified PME was determined by SDS-polyacrylaminde gel electrophoresis. A single protein band with a Mr. of 45 kDa (Figure 1) was obtained, suggesting the enzyme from kiwi *(Actinidia deliciosa)* fruit to be a monomer. From the results of present study it was fond that from ripened kiwi fruit PME can be purified with good recovery and specific activity. Nighojkar *et al.* (1994) also reported a monomeric form of PME of 54 kDa as determined by gel filtration chromatography and SDS-PAGE from germinating Vigna sinensis seeds. While working on ripe peach fruit, Glover and Brady (1994) observed three isoforms of PME with molecular weight of 34 kDa. Similarly MacDonald *et al.* (1993) reported PME of 35kDa and 33kDa from lemon fruit peel and endocarp, respectively. Ly-Nguyen *et al.* (2002 a, b) purified the Pectin methyl esterase from carrot and strawberry respectively with the help of affinity chromatography and reported that these are having a molecular weight of 50 and 45 KDa respectively.

Optimum temperature

To find out the optimum temperature of the enzyme, the final enzyme preparation was incubated for 45 min in water bath at temperature ranging from 25 to 80ºC. The enzyme was found to be temperature sensitive with optimum temperature at 30ºC. It could loss 50 per cent activity when incubated at 50ºC for 45 minutes. Pre-incubation of enzyme at 60 and 70 and 80ºC for 45 minutes of pre-incubation resulted in 64 and 79 per cent and total loss of activity, respectively. Nguyen *et al.* (2002 a,b) also purified the pectin methyl esterase from banana, carrot and strawberry respectively and found thermal sensitive although thermostable forms of PME have been also reported in lemon endocarp (MacDonald *et al.*, 1993), sweet cherry (Alonso *et al*., 1996) and persimmon (Alonso *et al*., 1997).

Optimum pH for enzyme extraction

Buffer of different pH (4.0 – 9.0) was used for extraction of PME. The enzyme activity as determined over a range of pH 4.0 to 9.0, increased

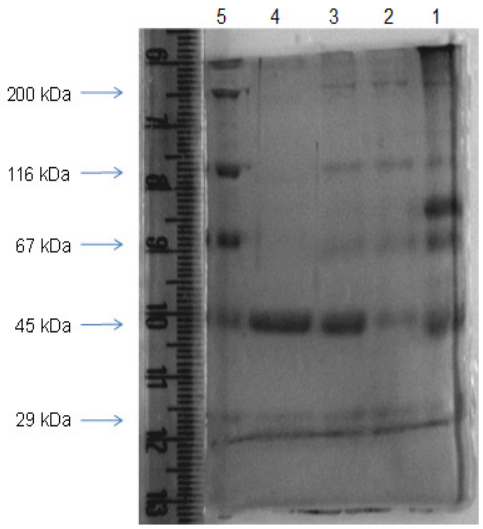

Figure 1. SDS-GAGE pattern of purified PME from kiwi *(Actinidia deliciosa)*. Lane 1: Crude extract, Lane 2: (30-80%) $(NH_4)_2SO_4$ fraction, Lane 3: DEAE sephadex column fraction, Lane 4: Purified enzyme, Lane 5: Standard Marker

from 0.33 units ml^{-1} at pH 4.0 to 2.09 units ml^{-1} at pH 7.5. Thereafter, enzyme activity decreased sharply, thus, clearly indicating, 7.5 to be the optimum pH for the enzyme extraction in case of kiwi fruit. Abu-Bakr *et al*. (2003) found optimum pH for PME extraction from guava fruit near to neutral pH of Tris-HCl buffer in presence of high salt concentration. Dixit *et al.* (2013) purified and characterized PME in datura *(D. stramonium)* over a range of pH (3–11), highest activity was observed between pH 7–10. The PME activity was recovered in the salt extract of the cell wall at slight alkaline pH, similar to most PMEs so far described (Balestrieri *et al.*, 1990; Micheli, 2001).

Effect of substrate concentration

The enzyme showed a typical hyperbolic response with increasing concentrations of substrate (0.1 to 1 per cent pectin) in an otherwise standard assay mixture revealing that it followed Michaelis-Menten kinetics. The enzyme activity increased with increasing concentrations of pectin until it reached a maximum at pectin concentration of 0.5 per cent in ripened kiwi fruit (Figure 2) of kiwifruit, above which the activity remained almost constant suggesting that enzyme got fully saturated at 0.5 per cent pectin. At ripened kiwi, Km value for the PME was found 0.164 mg ml^{-1} (Figure 3). Awad and Young (1980) reported similar Michaelis-Menten curve for substrate concentration for PME from avocado fruit. Km value for PME isolated from different sources has been reported to vary from 0.4 to 2.4 mg ml^{-1} (Hagerman and Austin, 1986; Giovane *et al.*, 1996). Km value of kiwifruit PME was found comparatively

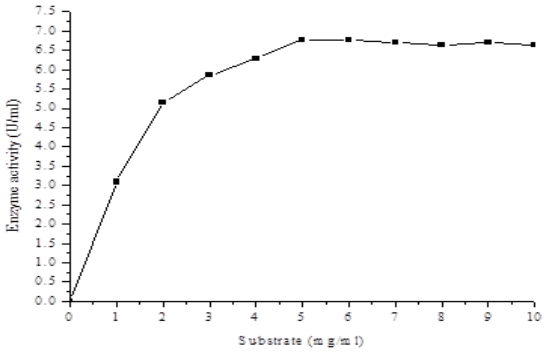

Figure 2. Effect of substrate (pectin) concentration on activity of purified PME of ripen kiwifruit

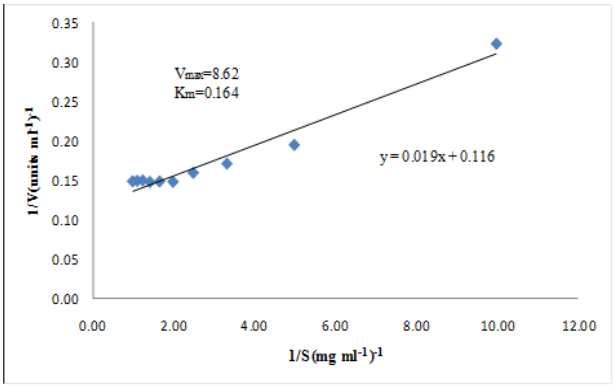

Figure 3. Line weaver-Burk plot for substrate (apple pectin)

lower then other sources. This indicates that ripen kiwifruit PME had higher affinity for the substrate then others.

Conclusions

Pectinases are one of the upcoming enzymes of fruit and textile industries. In present study we have reported the purification and charecterization of pectin methyl esterase enzyme (PME) from kiwi fruit (cv Hayward). Enzyme was purified about 20.75 fold and 37.90 recovery. Enzyme showed maximum activity at pH 7.5 with optimum temperature 30°C. Enzyme has molecular weight of 45 kDa. The enzyme showed a typical hyperbolic response with increasing concentrations of substrate revealing that it followed Michaelis-Menten kinetics. Km value of kiwifruit PME was found comparatively lower then other sources. This indicates that ripen kiwifruit PME had higher affinity for the substrate then others.

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