[IMP2 05] Purification and biochemical characterizations of β-galactosidase II from chilli (*Capsicum annuum* L.) fruit

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Introduction

Chilli or *Capsicum annuum* L. is among high value commodities of fruit vegetables in Malaysia. During its ripening, many physiological changes occur such as colour, hotness and physical attributes that define eating quality. However, softening problems during postharvest period has been quoted as a limited factor of consumers susceptibility. Ripening-associated softening in most of fruit including chilli leads to an increased susceptibility to physical damage during harvest period and/or pathogen attack during shipping and storage. In general, softening of many ripening fruits is associated with alteration in cell wall and middle lamella structure which reduces cell wall adhesion (Seymour & Gross, 1996). These changes include solubilization of cell wall protein involving the action of cell wall hydrolytic enzymes such as polygalacturonase, pectinmethylesterase, cellulase and β-galactosidase (Huber, 1983). Cell wall breakdown is caused by the concerted action of a number of proteins or enzymes including pectinmethylesterase (EC 3.1.1.15), which cleaves methyl ester groups from pectic polysaccharides; polygalacturonase, which hydrolyzes pectin; and β-galactosidase (EC 3.2.1.23), which removes the galactan side-chains from rhamnogalacturonan I (Brummell *et. al*., 1999).

Considerably attention has been given to fruit galactosidases, particularly β-galactosidase , as significant net loss of galactosyl residues from the cell wall is one of the best characterized events during ripening of many fruits such as tomato and papaya, (Pressey 1983; Ali *et al.,*1998). In bell pepper, β-galactosidase activity was most prominent in all stages of ripening and increased as the fruit ripened (Biles *et al*., 1997). However, the roles of β-galactosidase in our local chilli fruits are still not fully understood. Our objectives were to determine the activity of this softening enzyme in our local chilli ripening (cv. Kulai) and to purify this protein for biochemical characterizations.

Materials and Methods

Fruits and Sampling

Chilli fruits (cv. Kulai) were harvested at five ripening stages from a farm of Jabatan Pertanian Pontian, Johor Darul Takzim. These fruits were handled direct to laboratory, wash and rinsed by distilled water and dried at room temperature for overnight. Sampling was done by cut in 1cm sizes, froze by liquid nitrogen and kept in -80°C freezer until its used.

Enzyme Extraction

For determine the changes of β-galactosidase activity during chilli ripening, 100g of chilli tissues for each ripening stages were extracted and homogenized with citrate buffer 0.1M pH 4.6 (1% PVP, 10mM 2-mercaptoethanol) and then centrifuged at 17000 rpm for 30 minutes. Supernatant was filtered, clarified and used for enzyme assays (Pressey, 1983) and protein determinations (Bradford, 1976).

Activity assay & protein determination

The reaction mixture of β-galactosidase consisted of 0.520 ml of citrate 0.1M pH 4.1, 0.4 ml of BSA 0.1% and 0.4 ml of substrate β-D-galactopyranoside. Incubation was carried out for 10 mins at 37°C before 0.08 ml enzyme was added. After that, incubation was carried out again for 15 mins and the reaction stopped with Na_2CO_3 0.2M. The colour intensity of ρ-nitrofenol was measured at 415nm. One unit of enzyme is defined as the amount of enzyme required to liberate 1nmol ρ-nitrofenol per second (nkatal).

Protein contents of enzyme samples was determined by the method of Bradford (1976) using BSA as a standard.

Enzyme purification

500g of chilli tissues (red ripe stage) were used in this purification steps and all purification procedures were carried out at 4°C. Supernatant from this sample extraction was filtered, clarified and precipitated by 0-60% ammonium sulfate salt for 30 min. The precipitate was collected by centrifugation at 17000 rpm for 30 min, dissolved in acetate buffer 0.1M pH 5.2 and dialyzed against the same buffer for overnight. The dialyzate was then centrifuged. An aliquot was loaded onto a cation exchange column chromatography (30cm x 2.5cm) on CM-Sepharose CL 6B. The enzyme proteins were eluted by acetate buffer 0.1M pH 5.2 (0.1mM DTT) with stepwise gradient of 0.2M – 1.0M NaCl. Distinctly separable enzyme fractions were designated as A, B and C isoforms. The enzyme rich fractions- corresponding to B isoform was pooled, concentrated again with ammonium sulfate and then was used for further purification with gel filtration column chromatography. Isoform B was subjected to Sephacryl S-200 column (40cm x 2.5cm) and the activity proteins were eluted with acetate buffer 0.1M pH 5.2 (1mM DTT). Activity protein fractions was designated as β-galactosidase II isoform. The isoform was further purified with gel filtration chromatography column on Sephadex G-75 (30cm x 1.5cm) and eluted with the same buffer containing 1mM DTT. Each fractions collected were assayed for activity and protein determination as described previously.

Electrophoretic study

Purified proteins were fractionated by native-(Reisfeld *et al*., 1962) and SDS-PAGE (Laemmli, 1970). The separating gel for native- and SDS-PAGE were 12% and 15% acrylamide, respectively. The proteins were visualized after silver staining according to the method of Merril *et al*. (1981). A gel run in parallel for native PAGE was also subjected to activity staining for β-galactosidase using ρ-nitrophenyl substrate.

Native molecular weight determination

Molecular weight (MW) of enzymes were determined by elution from Sephadex G-100 (50 x 1.5cm) column with 0.05M potassium phosphate (pH7.0) buffer, which contained 0.15 M NaCl and 0.2% sodium azide. Standard proteins (SIGMA calibration kit) that include ribonuclease (13.7kD), chymotrypsin (25 kD), ovalbumin (43 kD), BSA (67 kD) and dextran blue (2000 kD) were used to calibrate the column.

Enzyme Kinetics

Enzyme kinetic was done as described in enzyme assay method using substrate concentration of 2-18 mM ρ-nitrophenyl-β-D-galactopyranoside.

Optimum temperature & pH, thermal stability

Optimum temperature was studied by using the method described in enzyme assay at the temperature range of 4°C-85°C while optimum pH was studied by the same method in the pH range of 2.0-6.5. For thermal stability, enzymes were incubated at the temperature range of 4°C-85°C for 10 min and cooled to 4°C before remaining activity was assayed as described in enzyme assay method.

Substrate specificity

All the procedure as described in Ali *et al*. (1998) with 13mM substrates of β- and α-D-galactopyranoside, β-glucopyranoside, α-mannopyranoside, α-arabinopyranoside and endogen substrates as 2% xylan, 1% spruce galactan and 1% arabinogalactan.

Effect cations & sugars on enzyme activity

Effect of cations were studied in enzyme activity assay, included magnesium chloride, sodium chloride, potassium chloride, mercury chloride, silver nitrate and EDTA in a different concentration range. The sugar effect examined included galactose, glucose, mannose, xilose, fucose and rhamnose.

N-terminal sequencing

β-galactosidase II protein was run on SDS-PAGE and the resolved protein were blotted from the gel onto a polyvinylidene difluoride membrane in 10mM 3 (cyclohexyamino)-1-propane-sulfonic acid buffer containing 10% methanol (pH 11) and submitted to the University of Southampton, UK for N-terminal sequencing.

Results

Activity profiles of β-galactosidase, α-galactosidase and α-mannosidase during chilli ripening

The activity profiles of three glycosidases at different stages of fruit ripening in chilli are presented in Fig.1. In the first stage of chilli ripening, these three glycosidases showed specific activity in a range of 3-7 nkatal/mg which α-mannosidase was the highest activity. After that, the activity of β-galactosidase increased significantly during fruit ripening with a peak of highest activity at red ripe stage (stage V) while activity of α-mannosidase increased with a peak activity at second stage but later decreased during ripening. α-Galactosidase activity was decreased significantly during fruit ripening. The profiles also showed that β-galactosidase activity was found ten times higher than α-mannosidase activity and three times than α-galactosidase activity during chilli ripening.

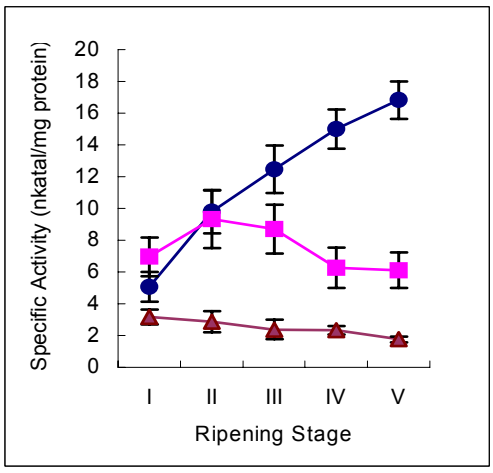

FIGURE 1 Activity of β-galactosidase (•), α-mannosidase (■) and α-galactosidase (▲) from chilli at different stages of fruit ripening. Data points are average ± S.D of four replicate samples

β-Galactosidase purification and characterizations

Chromatography of the crude extract of red ripe chilli on cation exchange cromatography (CM-Sepharose CL 6B) yielded three peaks of β-galactosidase activity and designated as peak A, B and C (Fig.2). Peak A and B were eluted in flow through fractions while peak C was eluted with 0.2M NaCl. Peak B was showed the highest activity than peak A and C even though the protein amount was low and so that for this paper, we reported the next study of peak B. Fractions of peak B was pooled and concentrated. The concentrated enzyme solutions was then loaded onto Sephacryl S-200 and eluted as a single peak of activity and named as β-galactosidase II (Fig.3). At this stage, most of the contaminant proteins were removed. The fractions of β-galactosidase II were further purified by chromatography on gel filtration of Sephadex G-75 and the result was considered as pure β-galactosidase II (Fig.4).

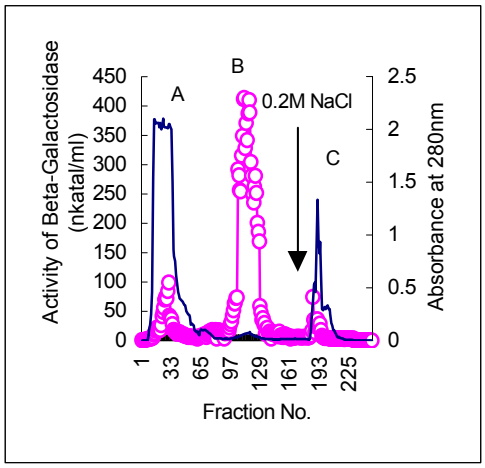

FIGURE 2 Elution profile of β-galactosidase activity fractionated from crude extracts of red ripe chilli fruits by use of a CM-Sepharose CL 6B. β-galactosidase activity (○) and absorbance at 280 nm (–)

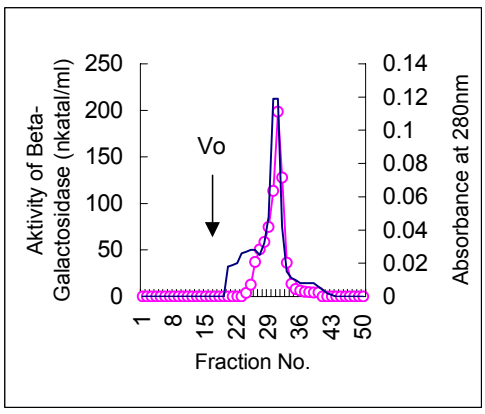

FIGURE 3 Elution profile of chilli β-galactosidase II activity (○) and absorbance at 280 nm (–) on Sephacryl S-200 gel filtration column.

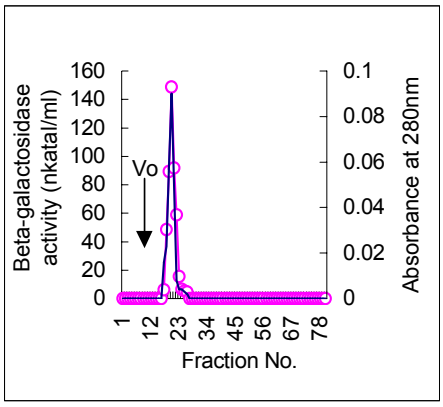

FIGURE 4 Elution profile of chilli β-galactosidase II activity (○) and absorbance at 280 nm (–) on Sephadex G-75 gel filtration column.

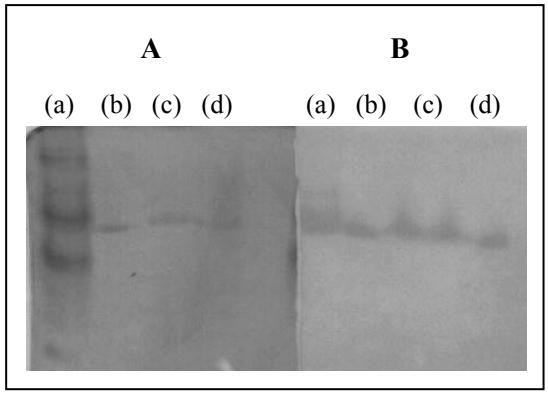

FIGURE 5 A native PAGE of β-galactosidase II protein from activity peak fractions of (a) crude extracts, (b) CM-Sepharose CL 6B, (c) Sephacryl S-200 and (d) Sephadex G-75. (A) silver staining and (B) activity staining.

TABLE 1 Purification of β-galactosidase from red ripe chilli (cv. Kulai). The starting material was 500g tissue and experiments were repeated at least three times.

Purification steps	Volume (ml)	Activity (nkatal)	Protein (mg)	Specific activity (nkatal/mg)	Recovery (%)	Purification fold
Crude extract	1165	15850.340	507.824	31.212	100	1
$(NH_4)_2SO_4$ & dialysis	131.2	5176.599	295.464	17.520	32.66	0.56
CM-Sepharose CL 6B	330	4464.735	8.585	519.895	28.15	16.66
Sephacryl S-200	80	177.324	0.909	195.076	1.12	6.25
Sephadex G-75	11	115.051	0.185	621.897	0.73	19.92

All the purification steps of β-galactosidase II was recorded in a purification table (Table 1). This table represents a typical purification scheme for chilli β-galactosidase. That in the final steps, Sephadex G-75 gel filtration chromatography increased further the specific activity and purified the enzyme for almost 20 fold. Figure 5 represents a native PAGE of β-galactosidase II protein in these purification steps and the protein also corresponds to the enzyme activity.

Analysis of β-galactosidase II isoform from fractions of final steps purification (Sephadex G-75) by electrophoresis on native PAGE showed that the enzyme appeared as a single protein band and it was also corresponds to the β-galactosidase enzyme as identified by activity staining (Fig.6). An estimated native molecular mass via gel filtration chromatography on Sephadex G-100 was about 75kD and analysis by SDS-PAGE revealed the presence of a single polypeptide band of 75kD (Fig.7). Apparent V_m and K_m values for β-galactosidase II were determined from Lineweaver-Burk plots. The V_m value was 196.078 nkatal and the K_m value with ρ-nitrophenyl-β-D-galactopyranoside as substrate was 5.157 mM. The temperature and pH optima for this enzyme were 45°C and 4.5 respectively. While heat stability showed that 50% activity loss in β-galactosidase II activity occurred at 60°C.

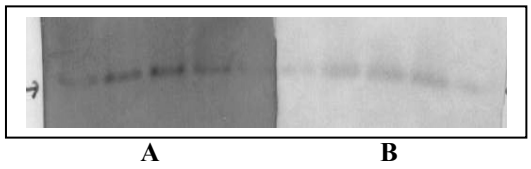

FIGURE 6 A native PAGE of proteins from β-galactosidase II activity peak fractions of Sephadex G-75 column. (A) silver staining and (B) activity staining.

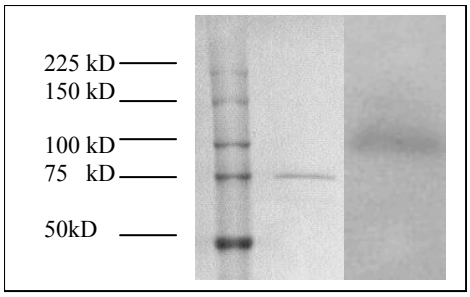

FIGURE 7 (A) SDS-PAGE and (B) activity staining of β-galactosidase after Sephadex G-75 column.

The activity of β-galactosidase II was not inhibited by 2M (final concentration) of Na^+, K^+ and 10mM of Ca^{2+}, Mg^{2+} but the addition of 0.5mM Hg^{2+} and Mg^{2+} to the reaction mixtures during β-galactosidase activity assay had completely inhibited the enzyme activity, while the addition of 5-10mM galactose was decreased 50% of enzyme activity. Results also showed that β-galactosidase II seemed to have no α-galactosidase, α-arabinosidase, β-glucosidase and α-mannosidase activity in substrate specifity test. β-galactosidase II showed active act against spruce galactan with consisted β-1,4 linked and no activity against arabinogalactan. N-Terminal amino acids from sequence of 75kD β-galactosidase II protein was performed on an Applied Biosystems Procise Sequencer and the sequencing had determined 15 amino acids. The amino acids sequence showed a similarity to translated sequence of capsicum and tomato β-galactosidase cDNA clone (Fig.8) and also others plant β-galactosidase.

Discussions

Changes in various glycosidase activity have been described for many fruits. Results of our study revealed that β-galactosidase is the major glycosidase increased significantly during chilli fruits ripening (Kulai cv.). This glycosidase also showed a high activity and increased during papaya and hot pepper ripening (Ali *et al*., 1998; Gross *et al*., 1986). But a study by Jagadeesh & Prabha (2002) found that β-hexoaminidase was showed high activity and increased significantly during bell pepper ripening while instead of that α-mannosidase was found to be present in abundance in bell pepper and hence was purified, followed by enzymological and immunological characterizations by Sethu *et al*. (1996). Nowadays, many purification and characterization research were done in fruits in a way to know a relationship of β-galactosidase in softening-associated ripening. Based on the increased of β-galactosidase activity in our early study, so that the enzyme purification and characterizations study is the best basic research in biochemistry of chilli ripening.

In our study, three β-galactosidase isoforms were separated from crude extracts of red ripe Kulai chilli and the predominant isoform, β-galactosidase II was purified by cation exchange and gel filtration chromatography. This was opposite with Biles *et al*. (1996) study which purified only one isoform of β-galactosidase from each green and red stage of bell pepper ripening. β-Galactosidase II appeared as predominant isoform in chilli with high activity, as a single polypeptide with apparently 75kD of molecular weight on SDS-PAGE and this comparable with the range of apparent molecular size of β-galactosidase from a number of others fruit. Of the reported of β-galactosidase proteins, β-galactosidase II papaya appeared as a single polypeptide of 67 kD (Ali *et al*., 1998) and coffee berry is claimed to consists of two subunit (Golden *et al*., 1993) while in others fruit (Ross et. al 1993; Carey *et al*., 1995) have revealed the presence of several other polypeptides in addition to the ripening-related β-galactosidase proteins.

β-Galactosidase II also could hydrolaze galactan with β-1,4 linked and therefore may be involved in the loss of galactose during chilli softening. However, in this study we are not yet act β-galactosidase II pure enzymes with isolated chilli cell walls or galactose-rich polysacharide. In Gross *et al*. (1986) study, the loss of galactose and arabinose residues from the cell wall, as well as observed modification of hemicellulose during ripening, seem to be unrelated to active polygalacturonase but the activity of β-galactosidase increased 50 fold from the immature green to the red ripe stage of hot pepper.

We conclude that these combination methods could be used to purified pure β-galactosidase II (75kD) from chilli crude extracts. The result is more convinced

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Caps_BAC10578       MMKSNNVLLVVLVICSLDLLVKANVSYDDRAIVINGKRKILISGSIHYPR 50
Caps_AAK40304       MMKSNNVLLVVLVICSLDLLVKANVSYDDRAIVINGKRKILISGSIHYPR 50
Tomato_II_T04340    MLRTNVLLLLVICLLDFFSSVKASVSYDDRAIIINGKRKILISGSIHYPR 50
Capsicum_protein    ----------------------NVSYDR--AVKNG-RKLK--------- 15
                                          .****     : ** **:
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FIGURE 8 Alignment of 15 amino acid sequences of β-galactosidase II protein (capsicum_protein) from red ripe stage chilli including those from the translated sequence of cDNA β-galactosidase capsicums (BAC10578, AAK40304) and tomato (T04340).The identical residue is indicated by (*), conserved substitution is indicated by (:) and semi-conserved substitution is indicated by (.)

with the N-terminal amino acid sequence of the 75 kD protein,which it showed a degree of homology with the translated sequence of capsicum and tomato cDNA clone which believed to encode a β-galactosidase. The present study of purification and biochemical characterizations of this predominant activity of β-galactosidase II may indicated that its possible play a major role in softening-associated ripening in chilli fruit.

Acknowledgements

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