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Papaya endoxylanase biochemical characterization and isoforms expressed during fruit ripening

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ABSTRACT

Papaya fruit ripening processes involve the coordinated action of several hydrolases that causes cell wall degradation. Endoxylanase participates in xylan or arabinoxylan modifications and its importance has been related to papaya softening. However, endoxylanase has been not fully characterized biochemically and kinetically. Semipurified endoxylanase from ripe 'Maradol' papaya fruit had an optimal temperature from 45 °C to 50 °C, a pH optimum of 5.5 against Remazol brilliant blue-xylan (RBB-Xylan) and enzymatic activity remained stable during 36 h at 45 °C. The activation energy of the enzyme was 25.5 kJ mol^{-1}, and the V_{max} at 32, 37 and 42 °C was 788.9, 888.9 and 1085.6 μg kg^{-1} s^{-1}, respectively. The K_m did not change as a function of temperature and was measured as 1.8 g L^{-1} and was within the range reported for other xylanases. Total proteins were extracted from color-break, half-ripe and ripe fruit. A pre-endoxylanase at 63.9 kDa was identified in the color-break fruit and an active endoxylanase at 32.5 kDa that was only found in ripe fruit, when the highest enzymatic activity was obtained. Immunodetection on two-dimensional gel electrophoresis (2DE) protein blots showed three isoforms of the pre-endoxylanase at color-break and ripe stages and, four isoforms in ripe fruit that were absent in color-break fruit. The biochemical and kinetic characteristics of the endoxylanase are crucial to our understanding papaya fruit softening.

1. Introduction

Papaya fruit ripening is a complex and irreversible process with many physiological and biochemical modifications that lead to changes in color, flavor, aroma, texture and firmness (Chen and Paull, 2003; Manenoi and Paull, 2007; Sañudo-Barajas et al., 2009). Ethylene-mediated softening during papaya ripening is a well-regulated phenomenon that involves structural changes such as depolymerization and solubilization of pectin, hemicellulose and cellulose, leading to fruit cell wall degradation (Sañudo-Barajas et al., 2009). The cell wall hemicellulose polysaccharides are composed by glucomannans, galactoglucomannans, galactomannans, xylans and arabinoxylans, that in association with the cellulose network provide the tensile strength to the cell wall (Brummell and Harpster, 2001). This network is degraded by hydrolytic enzymes during ripening causing overall fruit softening. Xylan, one of the main components of hemicellulose, consists of a complex heteropolysaccharide with a β-1,4-xylose backbone and branched with mono- and oligosaccharide residues (Brummell and Harpster, 2001). Various hemicellulases are required for its degradation such as the endo-1,4-β-D-xylanhydrolase (EC 3.2.1.8, endoxylanase) that hydrolyzes internal β-1,4-D-xylosidic linkages, producing short chains of xylose (Ming et al., 2008; Sañudo-Barajas et al., 2009; Thumdee et al., 2010).

The endoxylanase activity has been found during ripening of some fruit such as pear (*Pyrus* spp.) (Yamaki and Kakiuchi, 1979), tomato (*Lycopersicon esculentum* L.) (Barka et al., 2000), avocado (*Persea americana* M.) (Ronen et al., 1991), carambola (*Averrhoa carambola* L.) (Chin and Zainon, 1999), mango (*Mangifera indica* L.) (Bhagyalakshmi et al., 2002), and banana (*Musa sapientum* L.) (Phanayingphaisal et al., 2006). In papaya (*Carica papaya* L.),

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endoxylanase increases in activity during postharvest softening (Manenoi and Paull, 2007) and its activity is correlated with varieties having different softening rates (Paull and Chen, 1983; Chen and Paull, 2003; Manenoi and Paull, 2007).

Treatment with 1-methylcyclopropene (1-MCP), a competitive antagonist of ethylene reception, delays papaya softening, and its action is correlated with the absence or low endoxylanase activity in ripe fruit, and a "rubbery" flesh texture at the full ripe stage (Manenoi and Paull, 2007; Manenoi et al., 2007; Sañudo-Barajas et al., 2009). Endoxylanase expression appears to be regulated by ethylene as it is not detected by Western blot in 1-MCP treated fruit (Manenoi and Paull, 2007; Manenoi et al., 2007). A cDNA isolated from 'Sunset' papaya encodes a 65-kDa pre-endoxylanase, that is post-translationally modified to a 32.5-kDa mature endoxylanase as immunodetected by Western blot. The N-terminal sequence removed post-translationally contains a predicted carbohydrate-binding module (CBM), suggested to promote xylanase binding to papaya fruit cell wall and regulate its action throughout proteolytic cleavage (Chen and Paull, 2003; Manenoi and Paull, 2007).

Papaya fruit is rich on minerals, vitamins, fiber and antioxidants and, the 'Maradol' is the most economically important Mexican cultivar, whose marketing is limited due its rapid postharvest softening (Gayosso-García et al., 2010). An understanding of the hydrolytic enzymes involved in papaya fruit soften is crucial to developing approaches to control fruit softening rate. Endoxylanase has not been fully characterized from any papaya variety or in other fruit. The objective of this research was the endoxylanase semipurification from ripe 'Maradol' papaya, its biochemical (optimal pH and temperature, thermostability and activation energy) and kinetic (V_{max} and K_m as function of the temperature) characterization. In addition, we report several isoforms expressed during different ripening stages.

2. Materials and methods

2.1. Fruit

'Maradol' papaya fruit were purchased from a commercial orchard located in Tecomán, Colima, Mexico (latitude: 18.8765° and longitude: −103.859°). Fruit (three for each ripening stage) were randomly selected for uniformity in stage of external ripening and the absence of visible mechanical injury.

2.2. Fruit color and firmness

Fruit (three for each ripening stage) were grouped as color-break, half-ripe and ripe, based upon the commercial maturity indices of skin color and fruit firmness (Fig. 1 and Table 1). Lightness (L^*), chromaticity (*chroma*) and hue angle (*Hue*) were obtained at four equidistant points of the papaya skin (apical, equatorial and distal) with a chromameter (CM-2600d Konica-Minolta Inc., NJ, USA). In addition, fruit were longitudinally cut and the internal (pulp) color was determined at four equidistant points of the mesocarp. Hue angle (*Hue*) relates the values of a^* and b^* through the arctangent of $(b^* \cdot a^{*-1})$ of the CIELAB scale (McGuire, 1992) indicating the color-break fruit color during ripening ranged from green color (180°) to red (0°) with a lower value in ripe papaya fruit. The value of L^* represents brightness of the color ($L^* = 0$ for black and $L^* = 100$ for white), the value of *chroma* gives the intensity (from gray to pure hue) depending if the color is subdued or flashy so the value could be between 0 and 60 during the ripening process. Firmness (N) was determined at four points around the fruit equator (three fruit per treatment), using a penetrometer (Chatillon DFGS-100, TCD-200, 8 mm diameter tip, Largo, USA). Maximum force for tip insertion to a depth of 1.5 cm at speed of 5.2 mm s^{-1} was recorded. A decline in pulp firmness was observed as the fruit ripened (Table 1). Values for color and firmness were as previously reported for 'Maradol' papaya (Sañudo-Barajas et al., 2009; Gayosso-García et al., 2010).

Table 1
Firmness (N), light (L^*), chromaticity (*chroma*) and hue angle (*Hue*) values from 'Maradol' papaya fruit used to evaluate endoxylanase presence and its activity.

Ripeness	Firmness (N)	Skin color		
		L^*	*Chroma*	*Hue*
Color-break	144.3 ± 2.5a	44.6 ± 2.0a	34.3 ± 1.3a	121.6 ± 2.1a
Half-ripe	25.3 ± 2.3b	57.4 ± 3.0b	42.4 ± 2.5b	83.5 ± 4.6b
Ripe	13.5 ± 0.3c	55.5 ± 2.6b	45.9 ± 1.6c	70.3 ± 2.7c
Ripeness	Firmness (N)	Pulp color		
		L^*	*Chroma*	*Hue*
Color-break		60.6 ± 2.1a	31.8 ± 3.2a	80.1 ± 7.6a
Half-ripe		50.7 ± 2.1b	42.9 ± 0.1b	61.9 ± 2.8b
Ripe		48.4 ± 0.7b	45.0 ± 0.5c	60.5 ± 0.8c

N: firmness; L^*: light; *chroma*: chromaticity; hue angle (*Hue*) values. Values in the same column followed by different letter are statistically significant at a significance level of 0.05.

2.3. Semipurification of endoxylanase by ion exchange chromatography (CM-Sepharose)

Papaya endoxylanase semipurification was carried out according to the methodology described by Ali et al. (1998). Protein extracts from papaya (color-break and ripe fruit) were loaded onto an ion-exchange carboxymethyl Sepharose Fast Flow (CM-Sepharose) column (30 cm × 2.6 cm, GE Healthcare, Amersham Biosciences, NJ, USA) at flow rate of 21.7 μL s^{-1}. Proteins were eluted with 0.05 mol L^{-1} sodium acetate, pH 5.4 (buffer A), and fractions collected (6.5 mL). Each fraction was dialyzed against water for 8 h, lyophilized and resuspended in appropriated buffer for biochemical characterization.

2.4. Biochemical characterization of semipurified endoxylanase

2.4.1. Molecular weight determination

The native molecular weight of semipurified endoxylanase was determined using Sephacryl S-200 High Resolution column (52 cm × 2.6 cm, GE Healthcare, Amersham Biosciences, NJ, USA), previously equilibrated with buffer A and calibrated with a gel filtration standard kit: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B12 (1.35 kDa) (Bio-Rad) (Chen and Paull, 2003). Fractions (2.5 mL) were collected and their A_{280} and endoxylanase activity determined. Those fractions that contained endoxylanase activity were pooled and concentrated by ultrafiltration using centrifugal filters of 50, 30 and 10-kDa cutoff (Amicon, Millipore, MA, Billerica, USA). The pooled concentrated fractions were separated by SDS-PAGE and immunodetected as described below.

2.4.2. Glycoprotein staining

The presence of carbohydrate moieties of semipurified endoxylanase was carried out using a Pierce glycoprotein staining kit (Thermo Scientific, Rockford, USA) based in the general PAS (periodic acid-Schiff) method. The 12% polyacrylamide gel was directly stained according to kit instructions. The control proteins used were horseradish peroxidase (positive control) and soybean trypsin inhibitor (negative control).