

## Original Research Article

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## Partial purification and Characterization of Two Pectinases (Polygalacturonase and Pectin lyase) from Papaya Pericarp (*Carica papaya* cv. solo 8)

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### ABSTRACT

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Plant wastes could be a potential source of novel pectinases for use in various industrial applications due to their broad substrate specificity with high stability under extreme conditions. Therefore, the application conditions of novel pectinases (Polygalacturonase and Pectin lyase) from *Carica papaya* cv. solo 8 pericarp were optimized in this study. Those enzymes were partially purified by ammonium sulphate precipitation, dialysis and isoelectric focusing. The polygalacturonase and pectin lyase were partially purified 1.3 and 1.26 fold with a yield approximately 20 % and 24 %, respectively, following purification with isoelectric focusing. The specific activities of 3.62 and 0.176 UI/mg for polygalacturonase and pectin lyase enzymes were calculated, respectively. The optimal pH of 5.0 and 8.0 were obtained for the polygalacturonase and pectin lyase, respectively while an optimal temperature of 45°C was obtained for polygalacturonase and 50 °C for pectin lyase. Polygalacturonase and pectin lyase activities were enhanced by Na<sup>+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>, and Mg<sup>2+</sup> while EDTA has any effect at all concentrations on pectin lyase activity whereas EDTA was inhibitory to polygalacturonase activity. The results suggest that *Carica papaya* pericarp peels can be used for value added synthesis of pectinase, an important enzyme with numerous biotechnological applications.

### Introduction

Pectinases are responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in middle lamella and primary cell wall of higher plant. Depending on their mode of action, the enzymes hydrolyzing pectin are broadly known as pectinases, which include pectin methylesterases (E.C.3.1.1.11), polygalacturonase (PG) (E.C.3.2.1.15) and pectin lyase (PL) (E.C.4.2.2.10) (Kashyap *et*

*al.*, 2001). PG cleaves the polygalacturonic acid backbone of the pectin and reduces the average length of the pectin chains (Baron and Thibault, 1985). The pectin lyases (PL) act on pectins, oligomers and polymers of galacturonic acid by catalyzing the rupture between two galacturonic acid units by a  $\beta$ -elimination mechanism (Jeantet *et al.*, 2007). Pectinases have been used in various processes and industrials, which in the

degradation of pectic substances is essential (Favela-Torres *et al.*, 2005). Polygalacturonase and pectin lyase have a great commercial significance in industrial application (Kashyap *et al.*, 2001; Khan *et al.*, 2013; Tu *et al.*, 2013; Kumar and Suneetha, 2014). Whereas most industrial production of pectinases is limited to some species of bacteria, yeast (Gummadi and Panda, 2003; Jayani *et al.*, 2005; Combo *et al.*, 2012). Pectinase enzyme has been the focus of research for many years due to the potential and wide applications in various industrial processes. An increasing demand of pectinase has rendered the need of exploring under product capable of producing novel pectinases with improved activities. The screening realized by Yao (2013) on pectinases showed that their activities were two times higher in the pericarp than papaya pulp at mature stage presenting 1/8 yellow skin. This pericarp (peel) could be valorizing to production of commercial pectinases. This paper describes the partial purification and characterization of polygalacturonase and pectin lyase isolated from *Carica papaya* pericarp cv. solo 8.

## **Materials and Methods**

### **Plant material and enzyme extraction**

The survey carried on the peel (pericarp) of papayas (*Carica papaya* cv. solo 8). The ripe papayas are presenting 1/8 of the yellow skin studied in this work, were harvested from a farm near Thomasset (Azaguié), a village located at about 50 km of north of Felix Houphouet Boigny Airport, Abidjan (Côte d'Ivoire).

The papaya pericarp (50 g) was ground using a blender in 50 ml NaCl solution 0.9 % (w/v). The homogenate was subjected to sonication (4 °C) at 50 - 60 Hz frequency using a BANDELIN SONOPLUS HD 2200 for 10 min and then centrifuged at 10.000 g for 30 min at 4 °C. The supernatant was filtered

through cotton wool and was kept refrigerated and used as the crude extract.

### **Chemicals products**

Polygalacturonic acid, pectin from citrus fruits, bovine serum albumin (BSA) and Bradford reagent were supplied by Sigma-Aldrich. (St. Louis, MO, USA). The electrophoresis reagents on polyacrylamide gel were supplied by BIO-RAD. All chemicals and reagents used were analytical grade.

### **Enzyme assay**

The polygalacturonase (PG) activity was determined by a standard colorimetric method of Miller (1959) using 3,5-dinitrosalicylic acid (DNS). 900 µl of subtract (0.5 % of polygalacturonic acid prepared in 100 mM of acetate buffer pH 5) was mixed with 100 µl of enzymatic extract for a total of 1000 µl. Then the mixture was incubated at 40 °C for 15min. The reaction was stopped by adding 1.5 ml of DNS. After DNS was added the whole mixture was placed in a boiling water bath for 5 min. Finally, the mixture was let to cool down at ambient temperature and the absorbance was read at 540 nm using a blank (mixture without subtract) and D-galacturonic acid for the standard curve.

The galacturonase activity was expressed as the equivalent of galacturonic acid produced per milligram of protein per minute (µmol/mg/min).

The pectin lyase (PL) activity was determined using the method of Preiss and Ashwell (1963). An assay mixture (2.5 ml) consisting of a 100 mM Tris-HCl buffer pH 8.0, 10 mM CaCl<sub>2</sub>, 0.5 % pectin and enzyme solution was incubated at 40 °C for 1 h. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 548 nm.

One unit of pectin lyase activity was defined as the amount of enzyme present in 1 ml of enzymatic solution which released 1  $\mu$ M galacturonic acid for 1 min.

### **Protein estimation**

Protein concentrations and elution profiles from chromatographic columns were determined by Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard protein.

### **Partial purification of enzymes**

The Partial purification procedure was carried out in the cold room (4°C). Fifteen (15) ml of the enzymatic crude extract of the papaya (*Carica papaya* L. cv solo 8) pericarp were saturated with different concentrations of ammonium sulphate (20-80 %) and left with gentle stirring for 24 h in a refrigerator at 4°C. The mixture was then centrifuged at 6000 g for 30 min at 4°C with a refrigerated centrifuge (MIKRO 22R, Hettich). The precipitated proteins contained in the pellet were suspended either in 1 ml acetate buffer (0.1 M pH 5) or 1 ml Tris-HCl buffer (0.1 M pH 8) respectively for PG and PL.

The fractions containing the pectinases activities are pooled and subjected to dialysis. The dialysis extracts were immersed in a large volume of buffer (0.1 M acetate pH 7.5 or 0.1 M Tris-HCl pH 7.5) with continuous stirring for 16 h. The membrane having pores allowed small molecules such as ammonium and sulfate ions to cross, while not allowing the large protein molecules to pass through it. Every 4 hours the buffer solution was renewed.

This step made it possible to remove the excess of salt but also the substances of low molecular weight such as the pigments contained in the enzymatic raw extract. The

dialyzed extracts were subjected to an isoelectric-focusing technique. To start balancing the ion exchange membranes with a solution of phosphoric acid and sodium hydroxide overnight before using the membranes. This operation consisted in immersing the red anode in a solution of H<sub>3</sub>PO<sub>4</sub> (0.1 M), the black cathode in a solution of NaOH (0.1 M). Then the focusing chamber was prepared. This preparation consisted of mixing 100  $\mu$ l of dialyzed extract, 150  $\mu$ l ampholyte (Bio-lyte® 3/10, Bio-Rad Laboratories Hercules) and 2.75 ml of distilled water. Fill the 3 ml syringe with the sample and slowly charge the focus chamber. Then, 6 ml of 0.1 M H<sub>3</sub>PO<sub>4</sub> and 6 ml of 0.1 M NaOH were respectively added to the ventilation hole of the anode (red button) and cathodic (black button) assembly. Finally, the isoelectric focusing was carried out at 350 V, 3 mA, 25 W for 1 h. Once the IEF race was completed, the fractions were harvested as quickly as possible to avoid the diffusion of the separated proteins. PG and PL activities were assayed in each fraction to identify the isoelectric point of enzyme isolated from papaya pericarp cv solo 8.

### **Electrophoresis of proteins**

The partial purity of the enzymes was analyzed by native-polyacrylamide gel electrophoresis (Native-PAGE) (Laemmli, 1970). Silver staining was employed to identify protein bands.

### **Enzyme characterization**

#### **Effect of pH**

The influence of pH on the activity of purified polygalacturonase and pectin lyase were probed at different pH, either in 0.1 M sodium acetate buffer (pH 3.5 - 5.5), in 0.1 M sodium phosphate buffer (pH 5.5-7.5) or in 0.1 M Tris-HCl buffer (pH 7.0-9.0). PG and PL

activities were determined under standard conditions.

### **pH stability of enzyme**

To determine the pH stability, the polygalacturonase and pectin lyase were preincubated in 0.1 M acetate buffer (pH 3.5-5.5) and 0.1 M Tris-HCl buffer (pH 7.0-9.0) respectively at 4 °C for 1 h. Then assayed for polygalacturonase and pectin lyase activities in 0.1M acetate buffer (pH 5) and 0.1 M Tris-HCl buffer (pH 7.5) respectively.

### **Effect of temperature**

The effect of temperature on polygalacturonase and pectin lyase activities was determined by varying the temperature from 30 to 80 °C at an interval of 5 °C. The PG and PL activities were assayed as described above using polygalacturonic acid and pectin as substrates.

### **Determination of energy of activation and Q<sub>10</sub> values**

The energy of activation (E<sub>a</sub>) was estimated from the slope of Arrhenius plot obtained by plotting the Log of initial enzyme velocity against the reciprocal of the absolute temperature (Riet and Tramper, 1991).

Initial velocities were determined at different temperatures under standard assay conditions. Q<sub>10</sub> values were determined according to the method of Segel (1975).

### **Influence of metallic ions and EDTA**

The effects of metallic ions (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, Ba<sup>2+</sup>) and EDTA were determined by adding into the reaction system individually with the final concentration of 5 mM, 10 mM and 15 mM. The residual activity was determined under the standard condition as

describe above. The system without any additive was used as a control.

## **Results and Discussion**

### **Partial purification of polygalacturonase and pectin lyase**

A summary of the steps utilized to purify partially the pectinases from papaya cv. solo 8 pericarp was recorded in table 1. The process of pectinases partial purification was achieved with a protocol consisting of three steps. The crude pectinases were partially purified by ammonium sulphate precipitation, dialysis and isoelectric focusing. This first step ammonium sulphate precipitation 30 % not only concentrates the pectinases but also to eliminate some of the pigments contained in the raw extract (data not shown). The specific activities of polygalacturonase and pectin lyase in the step were evaluated as 2.4 UI/mg and 0.11 UI/mg, respectively (Table 1). Niharika and Abhishek (2014) also used 30 % ammonium sulphate precipitation as the first step for purification of apple pomace pectinases. Hammed and Alyaa (2010) used also ammonium sulphate precipitation as the first step for the purification of pectin lyase of *Erwinia carotovora* isolated from the spoiled potatoes. After ammonium sulphate precipitation the second step was dialysis. The increase in the specific activity of polygalacturonase (2.8 UI/mg) and pectin lyase (0.14 UI/mg) after dialysis could be explained by a loss of undesired proteins which interfere with the activity of pectinases (Undenwobele *et al.*, 2014). These two first steps of purification (ammonium sulphate precipitation and dialysis) of enzymes have already been reported by Ahmed *et al.*, (2016) in the purification of vegetable pectinases. The last step in the purification (Isoelectric focusing) of the polygalacturonase and pectin lyase isolated from the papaya pericarp cv. solo 8 made it possible to improve the

purification factor but also to determine the isoelectric point of the enzyme. This step made it possible to improve the specific activity of polygalacturonase (3.62 UI/mg) and pectin lyase (0.17 UI/mg). Polygalacturonase and pectin lyase were partially purified about 1.3 and 1.26 fold with final proteins of 2.86 mg and 3.56 mg, respectively (Fig. 1).

The specific activity of papaya pericarp cv. solo 8 polygalacturonase (3.62 UI/mg protein) obtained was less than polygalacturonase of *Achaetomium sp.* Xz8 (28.122 UI/mg protein) (Tu *et al.*, 2013) and polygalacturonase of *Aspergillus niger* MTCC 3323 (54.3 UI/mg protein) (Arotupin *et al.*, 2012). Otherwise, specific activity of papaya pericarp cv. solo 8 pectin lyase obtained was also less than pectin lyase of *Bacillus subtilis* BPLSY1 (58.85 UI/mg protein) (Al Balaa *et al.*, 2014). These differences in specific pectinases activities could be explained by the nature of the biological material used, by the technique used for purification and but also because these enzymes were partially purified.

Partial purities of polygalacturonase and pectin lyase were verified by performing native polyacrylamide gel electrophoresis. This electrophoresis shows a single protein task for polygalacturonase and pectin lyase but we remark a few protein tasks in band (Figure 2). This result indicates that those enzymes were partially purified.

### **Characterization of polygalacturonase and pectin lyase**

The results obtained demonstrated the maximum activities of polygalacturonase and pectin lyase partially isolated from papaya pericarp were observed at pH 5.0 and pH 8.0, respectively. Maximum polygalacturonase activity at pH 5.0 proves the acidophilic nature of polygalacturonase. The optimum pH of our findings was similar to the pectinase of

*Penicillium varidictum* RFC3 and *Aspergillus niger* (Silva *et al.*, 2002; Fahmy *et al.*, 2008), respectively. The range of pH at which polygalacturonase activity is maximal is between pH 3.5 and 6.0 (Fenghour *et al.*, 2002; Amande *et al.*, 2013). Polygalacturonase optimum pH was higher than that of purified polygalacturonase tomato (pH 4.4) (Verlent *et al.*, 2004) and lower than polygalacturonase of kiwi (pH 5.5) (Shouqing, 2014), *Aspergillus niger* and *Aspergillus flavus* (pH 5.5) (Deshmukh *et al.*, 2012) and *Bacillus sp.* KSM-P 410 (pH 7) (Jayani *et al.*, 2005). Polygalacturonase from *Carica papaya* pericarp could be favorable for the treatment in acid medium such as papaya juice (pH 5.6) (Tu *et al.*, 2013). Whereas maximum pectin lyase activity at pH 8.0 confirms the alkalophilic nature of pectin lyase partially purified. The optimum pH of the pectin lyase of the papaya pericarp CV solo 8 is identical to that of *Aspergillus niger* isolated from orange peel (Batool *et al.*, 2013) and *Aspergillus flavus* (Yadav *et al.*, 2008). On the other hand, it is lower than the optimum pH (pH9.5) of pectin lyase isolated from *Bacillus subtilis* BPLSY1 (Al Balaa *et al.*, 2014).

Polygalacturonase and pectin lyase activities were stable between pH 4.5 and 5.5 in sodium acetate buffer and pH ranging from pH 7.0 and 9.0, respectively. This pH range is almost similar to that of the purified polygalacturonase of *Penicillium sp.* CGMCC 1669 which is between pH 4 and pH 6 (Yuan *et al.*, 2011). These results suggest that this pH range (4.5-5.5) would be favorable for the expression of the enzyme. This zone could be ideal for clarification of fruit juices which have acidic pH, for the maceration of fruit and vegetables that have acidic pH. Concerning pectin lyase pH stability, these results suggest that working on this enzymatic activity in a pH range between 7 and 9; Could be ideal for treatment whose pH was in a basic medium.

**Table.1** Partial purification procedure of polygalacturonase and Pectin lyase from papaya pericarp cv. Solo 8

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification fold
Crude extract					
PG	326.93	680	2.08	100	1
PL	326.93	32	0.098	100	1
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation					
PG	85.8	206	2.4	30.33	1.15
PL	81.26	9.25	0.114	28.9	1.16
Dialysis					
PG	17.72	49.56	2.8	24.06	1.17
PL	17.90	2.5	0.140	27.03	1.23
Isoelectric focusing					
PG	2.86	10.35	3.62	20.88	1.3
PL	3.52	0.62	0.176	24.8	1.26

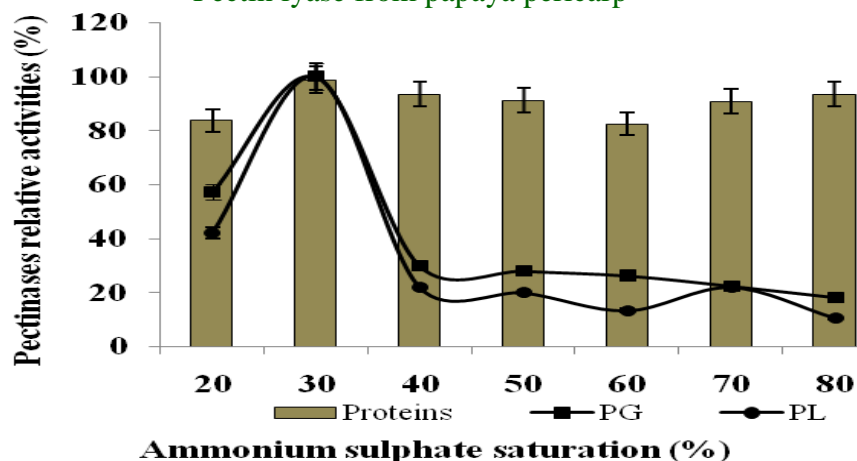
**Table.2** Some physicochemical characteristics of polygalacturonase and pectin lyase from papaya pericarp cv. Solo 8. Values given are the averages of at least three experiments ± SE

Physicochemical properties	Values	
	PG	PL
Optimum temperature (°C)	45	50
Optimum pH	5.0	8.0
pH stability	4.5-5.5	7.5-9
Activation energy (kJ/mol)	77.12 ± 0.03	68.77 ± 0.05
Q <sub>10</sub>	2.67 ± 0.1	1.61 ± 0.1
Activator agents	Mg <sup>2+</sup> , Ca <sup>2+</sup> , K <sup>+</sup> , Na <sup>+</sup>	Mg <sup>2+</sup> , Ca <sup>2+</sup> , Ba <sup>2+</sup> , K <sup>+</sup> , Na <sup>+</sup>
Inhibitor agents	Ba <sup>2+</sup> , EDTA <sup>a</sup>	-

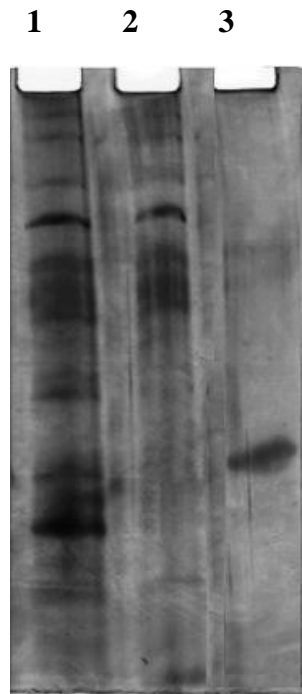
Values given are the averages of at least three experiments ± SE.

a: ethylene diamine tetraacetic acid.

**Fig.1** Effect of different ammonium sulphate saturation on polygalacturonase and Pectin lyase from papaya pericarp



**Fig.2** Native-PAGE of papaya pericarp. Lane 1, crude extract; lane 2, Polygalacturonase purified and lane 3, pectin lyase purified



The highest PG and PL activities were observed at 45 °C and 50 °C, respectively. Papaya pericarp polygalacturonase optimum temperature obtained in this work was in agreement with the polygalacturonase produced by gamma irradiated *Penicillium citrinum* (El- Batal *et al.*, 2013). This optimum temperature was lower than that of tomato polygalacturonase (Verlent *et al.*, 2004). Indeed, these authors obtained an optimum temperature of 55 °C. In addition partial purified *Carica papaya* pericarp pectin lyase optimal temperature was 50°C. This value was lower than pectin lyase from mango peel optimum temperature (60°C) (Amande *et al.*, 2013). From these results, polygalacturonase and pectin lyase isolated from *Carica papaya* could be used in food industry for processes requiring moderate temperatures. Those optimum temperatures (45°C and 50°C) were recommended for clarification and depectination operations.

This treatment must be carried at temperatures between 45 and 50 °C to avoid gelling during clarification operations (Grampp, 1977). These mesophilic enzymes could be coveted in the food industry during thermal operations (Table 2).

During thermal activation, polygalacturonase activation energy (Ea) and pectin lyase Ea were  $77.12 \pm 0.03$  kJ/ mol and  $68.77 \pm 0.05$  kJ/mol (Figure 4C and 4D) with  $Q_{10}$  values of 2.67 and 1.67, respectively. According to Lee and Wiley (1970) more Ea was high more the reaction was held quickly. The activation energy (Ea) value of the polygalacturonase of *Carica papaya* pericarp was four times lower than the value founded by Benen *et al.*, (2003) which is 324.17 kJ/mol on tomato polygalacturonase of variety CXD 199. These results suggest that the polygalacturonase isolated from *Carica papaya* pericarp cv. solo

8 reacted less rapidly than polygalacturonase isolated from tomato variety CXD 199.

The activities of polygalacturonase and pectin lyase partially purified were highly activated by  $Mg^{2+}$ ,  $Na^+$ ,  $K^+$  and  $Ca^{2+}$  (Figure 5). This result is agreement with Rexova-Benkova (1976) who reported that generally pectinases were activated by the presence of cations. The activation of pectin lyase activity by the  $Ca^{2+}$  was already been reported by several authors (Ren and Kermode, 2000; Arotupin *et al.*, 2012; Pedrolli and Carmona, 2014). According to Al Balaa *et al.*, (2014), in absence of the calcium ion ( $Ca^{2+}$ ) the pectin lyase activity was zero. Whereas, the polygalacturonase activity from *Carica papaya* pericarp was inhibited by 10 mM and 15 mM  $Ba^{2+}$ . However, the result was in contrary to pectinase enzyme from fruit and vegetable wastes and *Aspergillus niger* strain MCAS2, that  $Ba^{2+}$  ions found to enhance the pectinase activity (Ramachandran and Kurup, 2013; Khatri *et al.*, 2015). This suggests that the requirement of metals ions for the polygalacturonase activity vary depending upon their sources. In addition the inhibitory effect of barium ion would probably be a result of interactions between cations and peptide chains and not directly on the enzyme (Walter, 1991). Then EDTA was an inhibitor of PG activity but had no impact on the pectin lyase activity. Our results were similar to those of Arotupin *et al.*, (2012). These authors confirmed the inhibitory of EDTA in *Aspergillus niger* CSTRF polygalacturonase. On the other hand, our results are different from those of Whitaker (1984) showed that EDTA was generally an inhibitor of pectin lyase activity due to  $Ca^{2+}$  chelation.

In this study, the partial purification and some characteristics properties of polygalacturonase and pectin lyase extract from *Carica papaya* cv. solo pericarp were investigated. The polygalacturonase and

pectin lyase were partially purified successfully and showed reasonable stability to different conditions. From this characterization of papaya pericarp polygalacturonase and pectin lyase, it may be a potential candidate for industrial uses. Therefore, it can be employed in industries for hydrolysis of pectic biomass to utilizable bio-product. Future studies on pectinases isolated from *Carica papaya* pericarp should be devoted to the understanding the effect of heat treatment on these enzymes.

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