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Research Article
Partial Purification and Kinetic Properties of Polygalacturonase from *Solanum macrocarpum* L. Fruit

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Abstract

Background and Objective: Polygalacturonase [Poly (1, 4-α-D-galacturonide) glucanohydrolase, E.C 3.2.1.15] is a subclass of pectinase that hydrolyzes the glycosidic linkages between galacturonic acid residues in polygalacturonans. *Solanum macrocarpum* ripening prompts pectinase production. The objective of this study was to examine the kinetic properties of polygalacturonase from *Solanum macrocarpum* L. fruit. **Methodology:** The enzyme was partially purified by ammonium sulphate precipitation and gel filtration. Protein content, polygalacturonase activity and kinetic parameters were determined. **Results:** The protein content and polygalacturonase activity of the fruit juice extracts were 0.63±0.02 mg mL^{-1} and 45.96±6.31 U mg^{-1} protein, respectively. A 1.7 and 108.3 fold increase in enzyme activity was achieved by ammonium sulphate precipitation and gel filtration, respectively. The enzyme had a V_{max} of 76.92 unit mg^{-1} protein and K_m of 0.92 mg mL^{-1}. The pH profile of the enzyme showed three activity peaks at 3.0, 5.5 and 7.0. The enzyme was most active at pH 3.0 and showed optimal activity at 30°C. Rapid release of product was observed within the first 20 min of enzyme incubation. The Zn^{2+}, Ca^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+} and EDTA exhibited inhibitory effect on polygalacturonase activity whereas Mg^{2+} had stimulatory effect on the enzyme. **Conclusion:** It was concluded that the fruit of *Solanum macrocarpum* is a rich source of polygalacturonase. The enzyme is favourably comparable with that of a fungi source and could be further exploited for commercial production of the enzyme.

Key words: Polygalacturonase, *Solanum macrocarpum*, industrial application, partial purification, kinetic properties

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Enzymes are known effective catalysts and this characteristic promotes their use in several industrial products and processes[1]. Developments in biotechnology, especially genetics and protein engineering have led to the improvements in stability, economy, specificity and overall potential of industrial enzymes[2,3]. Pectin is a water-soluble fiber material that forms viscous gel-like suspensions. It is a high-molecular weight, complex polysaccharide consisting mainly of D-galacturonic acid units joined by α-(1-4) glycosidic bond[4,5]. It is abundant in the primary cell walls and middle lamella of the plant and a major contributor to the rigidity of plants and their fruit walls. Pectin is complexed with some of its free carboxyl groups partly or completely esterified to methanol and others are attached to calcium or magnesium to form a polygalacturonide[6]. Pectinases are enzymes of industrial importance. They are employed in extraction and clarification of fruit juice, winemaking, oil extraction as well as coffee making industries[7]. It is also used in the preparation of cellulose fibers for linen and in the manufacture of jute and paper. Pectinases are both plant and microbial in origin[8]. The enzyme group pectinase comprises of pectin methyl esterase, pectin lyase and polygalacturonase. The biotechnological potentials of these pectinolytic enzymes have drawn the attention of many researchers worldwide[2]. Polygalacturonase [Poly(1,4-α-D-galacturonide) glucanohydrolase, E.C. 3.2.1.15] is a pectin-degrading enzyme that hydrolyzes glycosidic linkages between galacturonic residues in polygalacturonans, a significant carbohydrate component of the pectin network that makes up the plant cell walls[5,6]. *Solanum macrocarpum* L. is widely distributed and cultivated in Nigeria and the African continent[9]. It is a tropical annual to perennial herb, shrub or subshrub plant, closely related to the 'eggplant' that is widely cultivated for food, medicinal purposes and as an ornamental plant[10]. The fruits of *Solanum macrocarpum* L. are largely oval shaped, with a creamish white colour, which could further look slightly yellow to orange when overripe. Its common names are 'Garden egg' and 'African eggplant' while the local names are 'igba' (yoruba), 'Gorongo' (Kanuri) and 'Anyara' (igbo)[9]. An understanding of the enzyme properties can help to regulate the ripening process of the selected fruit in order to prevent wastage and increase the shelf-life. Hence, this study was set up to investigate another source of enzyme, with probably new specificities that can possibly improve industrial efficiency. Occurrence of pectinolytic enzymes have been reported in a large number of bacteria and fungi[8]. Most commercial preparations of pectic enzymes are obtained from fungal moulds, such as *Aspergillus* sp.[11]. Although fruit ripening process triggers the production of pectinases, very little research has taken place on derivation of pectinase using the fruit itself as the source. So the present study was aimed to investigate the activity of polygalacturonase in the edible fruit of *Solanum macrocarpum*, partially purify the enzyme and determine its kinetic properties with a view to assess the potential use of the fruit extract as a source of the enzyme.

MATERIALS AND METHODS

Collection and identification of specimen: Edible fruits of *S. macrocarpum* were purchased from Oja market, Ota, Ogun State, Nigeria. The sample was identified by Dr. J.O. Popoola in the Department of Biological Sciences.

Chemicals: All chemicals were of analytical grade. The Na-K tartrate and ammonium sulfate where obtained from Klincent and Qualikems Laboratory, respectively. All other chemicals and reagents were obtained from Sigma-Aldrich Chemie.

Physical examination: On each day of the experiments, fruits selected for the day's experiments were visually and physically examined for its texture and colour.

Protein assay: Protein content was determined by using the Folin-Ciocalteau method[12]. Standard protein solution (Bovine serum albumin) of 0.1-1.0 mg mL^{-1} in duplicates was used. Absorbance was read against blank at 750 nm wave length. Protein concentration was measured in milligram per milliliter.

Polygalacturonase assay/sugar assay: The reducing sugar method as described by Miller[13] was used to assay for polygalacturonase (EC 3.2.1.15) activity. The galacturonic acid standard curve was prepared by making a serial dilution of 0.1-1.0 mL of 0.1% galacturonic acid in duplicate. Absorbance was read at 540 nm against blank. The reducing sugar released was expressed in galacturonic acid equivalent and expressed in units per milliliter. A unit of activity is defined as the amount of enzyme required to liberate 1 µmol of galacturonic acid per minute under the assay condition.

Partial purification of polygalacturonase: The crude extracellular enzyme was partially purified by the process of ammonium sulphate precipitation and gel chromatography as described by Chinedu *et al.*[9]. About 100 mL of the juice extract was dissolved in 20 mL acetate buffer (0.05 M, pH 4) and the enzyme was precipitated with ammonium sulfate to

80% saturation, by total dissolving of 56.1 g of ammonium sulfate. The solution was centrifuged at 6000 rpm for 10 min. The supernatant was carefully decanted and precipitate was dissolved in acetate buffer and left overnight at 4°C. Gel chromatography was used to fractionate proteins in a column chromatography (containing prepared Sephadex G-100 gel), equilibrated with acetate buffer 0.05 M (pH 4) at room temperature. Fractions of 5 mL were collected at a flow rate of 8 drops min^{-1}. These fractions were assayed for protein (at 280 nm) and polygalacturonase activity. Enzymatically active fractions were pooled and concentrated by freeze drying.

Properties of polygalacturonase

pH: The pH profile of the enzyme was determined by varying the pH of the reacting mixtures between 3.0 and 9.0. These substrates were prepared in two buffer solutions: 0.2 M acetate-NaOH (pH 3.0-7.0) and 0.1 M Tris-HCl buffer (pH 8.0-9.0).

Temperature: The temperature profile of the enzyme was determined by incubating the enzyme with substrates for 30 min at various temperatures between 30-100°C. The enzyme activity values were determined.

Time course: The time course of the enzyme was determined by measuring the enzyme activity at different periods of incubation under the standard assay conditions of pH 3.0 and temperature 30°C. Total reducing sugars (galacturonic acid equivalent) released by the enzyme per time was monitored at 10 min intervals for 70 min.

Effect of substrate concentration: The effect of various substrate (polygalacturonic acid) concentrations (0.2-1.0 mg mL^{-1}) on the enzyme activity was studied under the standard assay conditions of pH 3.0 and temperature 30°C. The Michaelis Menten constant (K_m) and maximum velocity (V_{max}) of the enzyme were obtained using the reciprocal plot (Lineweaver Burk plot).

Effect of metal ions and EDTA: The effect of divalent cations and ethylene diaminotetraacetic acid (EDTA) was determined on the enzyme-substrate mixture containing 2.0 mM salts of the respective cations at the standard assay conditions for 30 min. The salts included Pb $(C_2H_3O_2)_2$, $MgSO_4$, $CaSO_4$, $CuSO_4$, $FeSO_4$ and $ZnSO_4$. Effect of cation chelator, EDTA, on the enzyme activity was also determined by including 10 mM of EDTA in enzyme-substrate mixture.

Data analysis: All assays were in triplicates and results were expressed as mean, unless otherwise stated.

RESULTS

Phenotypic characteristics of fruit sample: The phenotypic characteristics of *S. macrocarpum* L., fruit sample was used. Table 1 shows that the fruit was edible, hard, undulating with an oval shape. Results recorded in Table 2 for protein concentration, reducing sugar and polygalacturonase activity were reported as 0.63±0.02 mg mL^{-1}, 1.58±0.04 mg mL^{-1} and 45.96±6.31 U mg^{-1} protein, respectively.

Protein concentration of eluates: Figure 1 shows the elution profile of the various 5 mL fractions (numbered 1-40) containing protein collected at a flow rate of 8 drops per minute. These fractions were assayed for protein (at 280 nm) using gel filtration (Sephadex G-100).

Polygalacturonase activity of protein fractions: Figure 2 shows elution profile of the polygalacturonase activities of each protein fraction, with fractions 36 and 37 showing highest activities. Fractions 4, 29, 30, 36 and 37 were pooled together.

Purification of polgalacturonase: The purification steps, yield and purification fold of polygalacturonase from *Solanum macrocarpum* fruit are recorded in Table 3. Enzyme purification fold of 1.7 and 108.3 was observed using ammonium sulphate precipitation and gel filtration respectively are presented in Table 3.

Properties of polygalacturonase: The effect of pH, temperature, time course, substrate concentration, metal ions and EDTA on polygalacturonase activity were examined and shown in Fig. 3-7 and Table 4, respectively.

Table 1: Physical description of *S. macrocarpum* L.

Name	*Solanum macrocarpum* L.
State	Edible
Peel color	White with thin green stripes
Pulp color	Whitish-brown (cream)
Texture	Undulating
Hardness	Very hard
Fruit shape	Oval

Table 2: Protein concentration, reducing sugar and polygalacturonase activity

Name	*Solanum macrocarpum* L.
State	Edible
Protein concentration (mg mL^{-1})	0.63±0.02
Reducing sugar (mg mL^{-1})	1.58±0.04
Polygalacturonase activity (U mg^{-1} protein)	45.96±6.31

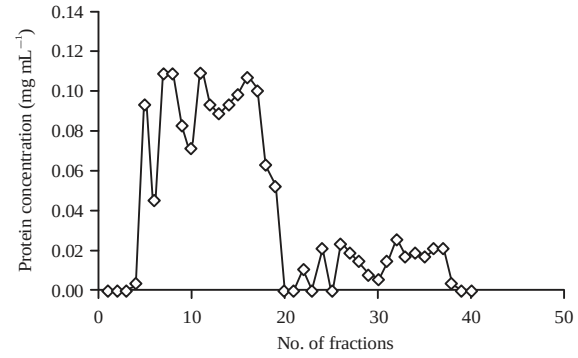

Fig. 1: Elution profile of protein fractions of *S. macrocarpum* fruit (Sephadex G-100)

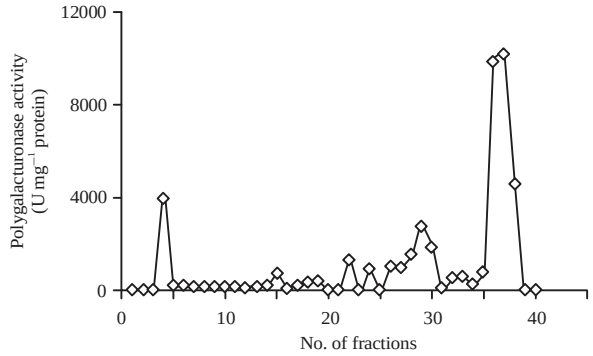

Fig. 2: Elution profile of polygalacturonase activity of *S. macrocarpum* fruit (Sephadex G-100)

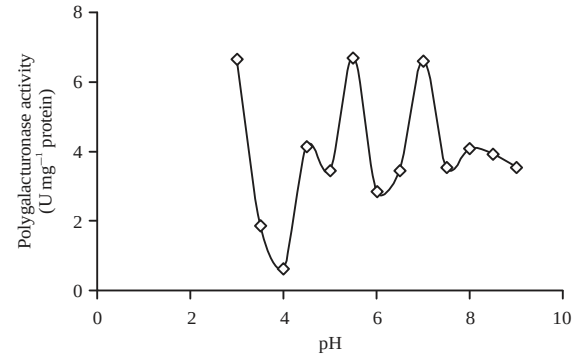

Fig. 3: Effect of different pH (3.0-9.0) on the polygalacturonase activity of *S. macrocarpum* L., fruit

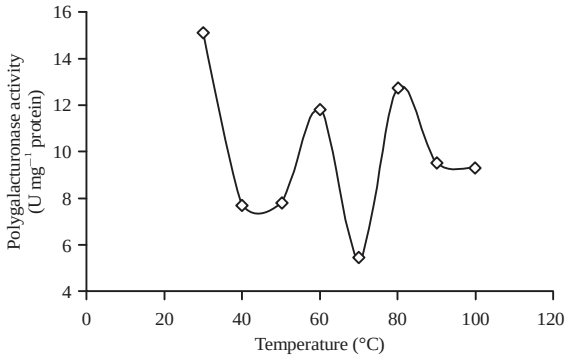

Fig. 4: Effect of different temperature (30-80°C) on the polygalacturonase activity of *S. macrocarpum* L., fruit

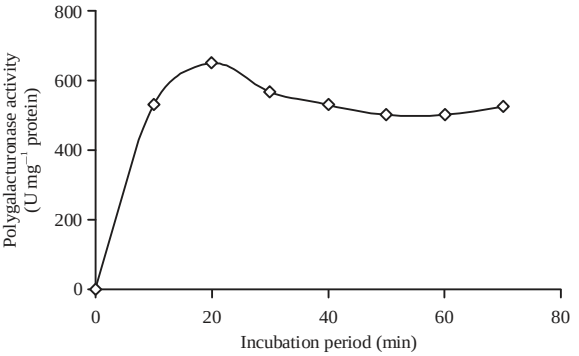

Fig. 5: Time course of polygalacturonase activity in *S. macrocarpum* L., fruit juice extract

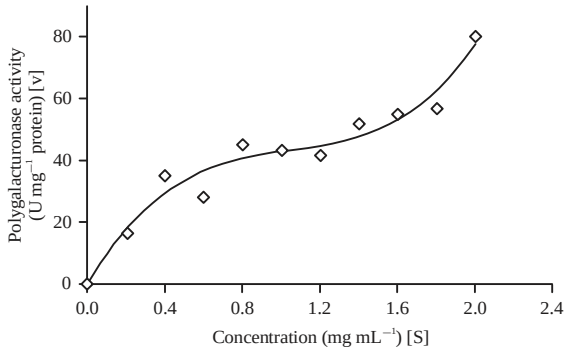

Fig. 6: Michaelis-Menten plot showing the effect of polygalacturonic acid concentration on polygalacturonase activity of *S. macrocarpum* L., fruit

Table 3: Purification of polygalacturonase from *Solanum macrocarpum*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude enzyme extract	6317	67.00	94.28	100.00	1
$(NH_4)_2SO_4$ precipitation	1243	7.70	161.45	19.68	1.7
Gel filtration (Sephadex-G100)	1128	0.11	10211.47	17.85	108.3

Effect of pH: *Solanum macrocarpum* polygalacturonase activity at different pH (3.0-9.0) in Fig. 3 shows three activity peaks (3.0, 5.5 and 7.0) with 3.0 being the highest activity peak obtained for the enzyme.

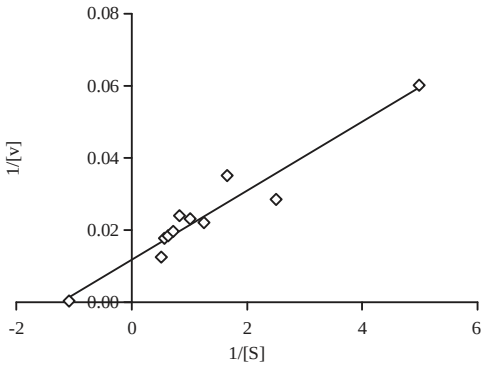

Fig. 7: Lineweaver Burk plot of the polygalacturonase of *S. macrocarpum* L., fruit

Table 4: Effect of metal ions and EDTA on polygalacturonase activity of *Solanum macrocarpum* fruit

Metal ions and EDTA	Activity (%)
Control	100.00
EDTA	95.32
Pb^{2+}	89.04
Ca^{2+}	85.14
Fe^{2+}	95.80
Cu^{2+}	97.72
Mg^{2+}	104.02
Zn^{2+}	84.19

Temperature profile: The temperature profile as recorded in Fig. 4 shows that at different temperatures (30-80°C), *S. macrocarpum* polygalacturonase showed optimal activity at 30°C with over 50% activity at 60 and 80°C.

Time course of polygalacturonase: Incubation of the enzyme at different time are presented in Fig. 5, which shows galacturonic acid was released rapidly in the first 20 min followed by a decline thereafter up to 50 min.

Effect of substrate concentration on polygalacturonase activity: The relationship between the polygalacturonase activity of *S. macrocarpum* L., fruit and various substrate concentrations is reported in Fig. 6, where a rapid increase in the activity of the enzyme was observed as the substrate concentration was increased from 0.2-1.0 mg mL^{-1}. Thereafter, subsequent increase in substrate concentration had little effect on the rate of enzyme activity. The maximum velocities (V_{max}) and Michaelis-Menten constant (K_m) of the enzyme from the Lineweaver Burk plot was extrapolated to be 76.92 U mg^{-1} protein (15.38 µmol min^{-1}) and 0.92 mg mL^{-1}, respectively.

Effect of metal ions and EDTA on polygalacturonase activity: The Zn^{2+}, Ca^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+} and EDTA had an inhibitory effect on the polygalacturonase activity while Mg^{2+} had a stimulatory effect of 4.02%. Zinc indicated the highest inhibitory effect (15.81%) and copper indicated the lowest inhibitory effect (2.28%).

DISCUSSION

Pectinases can be regarded as the most important enzyme of the fruit juice industry[14]. Its production is crucial for the successful clarification and hydrolysis of pectin derived materials[6]. Pectinases are of microbial and plant origin, however, our focus is on the plant origin. Microbial species, such as *Aspergillus* sp., are major sources of commercial pectinases[5]. Only those microbes that produce a substantial amount of extracellular pectinase have been exploited commercially[8]. The researchers carried out a preliminary study on the overripe and ripe fruits of *S. macrocarpum*, to determine the appropriate state at which the fruit had polygalacturonase activity at its maximum. Results showed that polygalacturonase activity was highest in the ripe (edible) fruit of *S. macrocarpum* L. Hence, the ripe fruit was used for this study. This informs us of the point at which this enzyme is produced and this could be exploited for storage and processing. The possible sigmoidal curved derived from the Michaelis-Menten plot, indicates the allostericity of this polygalacturonase produced by *S. macrocarpum*. An allosteric enzyme is characterized with more than one binding site, where the binding of substrate on one active site affects the properties of other active sites in the same enzyme molecule. A possible outcome of this interaction between subunits is that the binding of substrate to one active site of the enzyme facilitates substrate binding to the other active sites[15]. The maximum activity (V_{max}) and K_m of polygalacturonase obtained from the crude enzyme extract (juice) of *S. macrocarpum* fruit, compared favorably with that of *Tetracoccosporium* sp., a fungal source of polygalacturonase[2]. The polygalacturonase activity of this study is observed to have a lower K_m, inferring that the substrate concentration needed for V_o to attain half its maximum velocity is lower. Polygalacturonase of *S. macrocarpum* fruit can work effectively at very low concentrations of the substrate, a necessary and important consideration for industrial processing and production. The effect of different pH values on polygalacturonase activity in *S. macrocarpum* fruit showed three activity peaks. The optimal pH for different pectinases has been reported to vary from 3.8-9.5, depending upon the type of enzyme and the source[16]. The polygalacturonases of *Aspergillus niger* and

Tetracoccosporium sp., were reported to have their optimal activities at pH 5.0 and 4.3[2,17]. The implication is that the polygalacturonase can tolerate variations in pH and function effectively at both acidic and neutral environments. This makes the enzyme well suited for the acidic environment as well as neutral environment of most industrial processes. The optimum temperature for the enzyme was 30°C, it also retained over 50% of its activity at 60 and 80°C under assay conditions. This may be as a result of the adaptation to hot-humid climate where the fruits were obtained. Shubakov and Elkina[17] reported an optimal temperature of 50 and 60°C for the polygalacturonase enzyme of *Aspergillus niger* and *Penicillium dierckii*, respectively. Aminzadeh *et al.*[2] however reported that of *Tetracoccosporium* sp., to be 40°C. The higher the optimal temperature obtained for the enzyme the better the heat stability. This is a desirable quality, especially in industrial processes where thermal treatment may be inevitable. Some metals ions, Zn^{2+}, Ca^{2+}, Mn^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+} and EDTA exhibited slight inhibitory effect (2.0-6.0%) on the polygalacturonase activity whereas Mg^{2+} had about 4.02% stimulatory effect on the enzyme. The effects of metals and EDTA on polygalacturonase vary depending on the source and type of enzyme. The report by Aminzadeh *et al.*[2] showed that extracellular polygalacturonase produced by filamentous fungus *Tetracoccosporium* sp., was inhibited by Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and SDS, however, EDTA and Co^{2+} stimulated the activity. Polygalacturonase from fruit shows a higher ability to withstand metal inhibition than others from literature. Also an addition of Mg^{2+} would enhance its activity, this gives us an inclination to its mechanism of action. Partial purification of the fruit juice extract of *S. macrocarpum*, showed that there was a 1.7 and 108.3 fold increase in enzyme activity by $(NH_4)_2SO_4$ precipitation and gel filtration.

CONCLUSION

It was concluded that the fruit produces extracellular proteins with polygalacturonase activity comparable to commercially available polygalacturonase preparations. The results of the study showed higher V_{max}:K_m values, when compared to other sources of the enzyme from literature. Hence, polygalacturonase of *S. macrocarpum* fruit is a potential source for industrial production of an equally efficient enzyme. Further study is needed to investigate the allostericity of this enzyme. It is recommended that further studies be done to help optimize the industrial use of enzymes from plants in order to improve processes and conserve fruit and vegetable waste.

SIGNIFICANT STATEMENTS

The maximum activity (V_{max}) and K_m of polygalacturonase obtained from *S. macrocarpum* fruit, can be compared with that of *Tetracoccosporium* sp. Polygalacturonase of *S. macrocarpum* fruit can work effectively at very low substrate concentrations, tolerate variations in pH and function effectively at both acidic and neutral environments. The optimum temperature for the enzyme is of desirable quality and shows a higher ability to withstand metal inhibition than others from literature.

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