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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF SEVERAL CARBOHYDRASES (POLYGALACTURONASE, SUCRASE AND B-GALACTOSIDASE) FROM THE SHRUB, *CALOTROPIS GIGANTEA*

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ABSTRACT

The shrub, *Calotropis gigantea* was found to be an excellent source of polygalacturonase, sucrase and β-galactosidase containing about 34000 ± 2000, 6500 ± 300, and 600 ± 100 units of enzyme respectively, per 100 mL latex. The stem bark contained (units / 100 g of fresh tissue) about 11500 ± 500 (pectinase), 975 ± 20 (sucrase) and 1000 ± 100 (β-galactosidase). The enzyme polygalacturonase was acid stable and readily hydrolyzed crude guava, apple and citrus pectin optimally at 45- 50°C. The enzyme retained about 70 % of its original activity when incubated in buffer of pH 2.0 over a period of 3 h. The enzyme was partially purified by ammonium sulphate precipitation followed by gel filtration chromatography. Polygalacturonase was insensitive to heavy metals like $Pb2^{+}$, Cd^{2+}, Ag^{+} but Hg^{2+} inhibited its activity. Ca^{2+} and Zn^{2+} slightly improved the enzyme activity. DTNB, iodoacetic acid and iodoacetamide did not inhibit polygalacturonase activity suggesting the non-thiol nature of enzyme protein. Sucrase showed excellent pH stability (pH 2 - 8) and could hydrolyze raffinose and inulin (slight). The latex also exhibited slight amylolytic and xylanolytic activities.

Keywords: Pectinase, Fruit Juice Clarification, *C. gigantea*, Crude Pectin, Sucrase

INTRODUCTION

Though food and pharmaceutical industries prefer the use of plant enzymes instead of microbial enzymes for various reasons **[1]**, yet plants have never been studied seriously as source of commercial enzymes except for malt amylase, papain and bromelain obtained from barley malt, papaya latex and pineapple respectively. Large amount of plant proteases (bromelain, papain etc.), malt amylase are consumed by brewing and food processing industries as well as by pharmaceutical houses as digestive enzyme **[2, 3]**. We reported earlier that a non-cereal, non-leguminous plant *Tinospora cordifolia* Miers, (family Meninspermaceae), produces substantial amount of extracellular saccharifying amylase and acid stable disaccharidases (sucrase, maltase, isomaltase) in the plant body **[4, 5]**.

Pectinase is a well known term for commercial enzyme preparation that break down pectin (a polysaccharide substrate, found in the cell wall of plants) by splitting polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages. This process softens the cell wall and increase the yield of juice extract from the fruits. Microorganisms and plants are the two major sources of pectinase but considering the yield and economic point of view microbial sources have become extremely important. Food industries usually use fungal pectinase as fungi are the potent producers of pectic enzymes **[6]**. Over the years, pectinases have been used in several conventional industrial processes such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater containing pectinacious material, etc.

Calotropis gigantea Linn. (family Asclepiadaceae), a glabrous laticiferous shrub, abounding in milky juice, commonly known as the swallow-wort or milkweed **[7]** is native to India and grows well throughout the country on unconditioned soil producing huge biomass throughout the year. In India the plant is called by various names: Arka, Mandara in Sanskrit; Madar in Hindi; Akanda in Bengali; Akado in Gujarati; Mandaramu in Telegu. *Calotropis* is used as a traditional medicinal plant with unique properties **[8]**. Charak recommended the root bark in piles and leaves to cover boils while Sushrut mentions its use in ear-ache and asthma **[9]**. Traditionally *calotropis* is used alone or with other medicinal herbs to treat common disease such as fevers, rheumatism, indigestion, cough, cold, eczema, asthma, elephantiasis, nausea, vomiting, and diarrhea **[10]**. According to Ayurveda, the plant is a

good tonic, expectorant, depurative, expectorant, and laxative **[11]**. The latex is also used to treat depilatory, asthma, enlargement of spleen and liver, relief from painful joint swellings **[12]**.

The present study reports the presence, partial purification and characterization of an over produced, acid stable polygalacturonase (170000 ± 5000 / 100 g of dry latex) from the shrub, *Calotropis gigantea* capable of hydrolyzing crude pectin from guava, apple and citrus peel. The enzyme also exhibited xylanolytic and amylolytic activities, thus making the enzyme cocktail an excellent choice to be used in fruit juice clarification. The enzyme cocktail was also an excellent source of sucrase (35000 ± 2500 U/ 100 g of dry latex) and β-galactosidase.

MATERIALS AND METHODS

Materials

Pectin was purchsed from SRL, India. Sucrose, maltose, isomaltose, maltotriose, glycogen (oyster shell), pullulan (from *Aureobasidium pullulans*), levan, raffinose, melizitose, inulin, soluble starch (potato), bovine serum albumin (BSA, Fraction-V, A3059), dinitrosalicylic acid (DNSA), Bradford reagent were all purchased from Sigma chemicals, USA. p-nitrophenyl-α-D-glucopyranoside, o-nitrophenyl-β-D-galactopyranoside was purchased from SRL, India. Trehalaose was a product of British Drug house (BDH). Glucose oxidase-Peroxidase (GOD-POD) reagent was a product of Span Diagnostics, India. P-chloro mercury benzoic acid (PCMB), iodoacetic acid, iodoacetamide, 5,5 dithio-2-nitro(bis)benzoic acid (DTNB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were purchased from Sigma chemicals. Biogel- P100 was purchased from BioRad. All other chemicals used were of chemically pure grade.

Extraction of Enzyme

Enzyme was extracted as mentioned in the patent **[4]**. Mature plants (2 – 2.5 m height) growing wild in different fields were selected and a sharp incision was given at the first and second node region of the stem with a sharp blade. Latex exuded was collected in centrifuge tubes (15 mL capacity). Latex was collected in early morning. Latex was also collected from the leaves and fruits by giving a sharp incision. The latex collected was centrifuged which resulted in three separate layers: the upper solid (less dense) and bottom solid (more dense) and the middle liquid part. The middle liquid part was taken out and used a source of crude enzymes. The liquid latex was spread on a glass plate and kept in a hot air oven at 40ºC for 4 h. The dry latex powder was scrapped from the glass plate and stored

in air tight containers at 4ºC. The dry powder was also used as source of crude carbohydrases.

Alternatively, mature plants growing wild in different fields were collected and sorted to eliminate infected part, if any. Stem parts having a thickness of more than 2.5 cm were freed from leaves and thoroughly washed with water. The greenish stem-bark was peeled off from the whole stem using a sharp peeler. The stem-bark pieces (50 g) obtained was immersed immediately in 100 mL of 0.1M acetate buffer, pH 5.0 (kept in a water bath at 45 ^{0}C) for 30 min. The mixture was blended (4-5 min) in a warring blender to obtain a paste of the biomass. The mass was squeezed through a nylon cloth to obtain a greenish liquid. The filtered liquid was kept overnight at 4ºC when precipitation of a white mass of gummy material takes place. The clear liquid (95 mL) was filtered out under suction and ultra-filtered using PM-10KDa membrane. The concentrated liquid (20 mL) was further lyophilized to dryness and used as a source of crude pectinase and disaccharidases.

Determination of Storage Stability of Plant as a Source of Enzyme

The storage stability of source and enzyme was determined in the following ways: 1) Washed fresh stem (100 g) were cut into 5 - 6 cm long pieces, and stem-bark was peeled off as mentioned above. The stem-bark pieces were kept either at 0 - 4ºC in sealed polyether bags or lyophilized to dryness and stored at room temperature in air tight containers. 2) Fresh latex was collected as mentioned above and stored at at 0 - 4ºC in air tight containers. 3) Dry latex powder (prepared as mentioned above) was kept in sealed containers at 0 - 4ºC. 4) Enzyme extracted by blending the stem-bark (as mentioned above) was stored at 0-4ºC. The enzyme solution was also ultra-filtered and lyophilized to dryness. The dry solid was stored in sealed containers at 0 - 4ºC. 5) Fresh latex was subjected to ammonium sulphate precipitation (80 % saturation) followed by dialysis and lyophilization. The lyophilized latex protein was stored at 0 - 4ºC.

Plant materials and enzyme preparations stored under all these above mentioned conditions were assayed for polygalacturonase, sucrase and β-galactosidase activities at regular time intervals till 6 months of study.

Partial Purification of the Enzymes

C. gigantea latex (20 mL) was subjected to ammonium sulphate (80 % saturation) precipitation. The sample was kept overnight at 4ºC and centrifuges at 10,000 rpm for 10 min to obtain the enzyme-protein pellet. The

pellet was finally dissolved in 0.1M acetate buffer, pH 5.0 containing 10 mM $CaCl_2$ and dialyzed for 24 h against the same buffer. Enzyme solution (1 mL) containing 2 mg of protein was charged each time on BioGel P-100 column (2.0 cm x 65 cm), pre-equilibrated with the above mentioned buffer. Fractions (2.0 mL each) were collected until the column was free from 280 absorbing material. Elution of protein was monitored at 280 nm. Each fraction was assayed for polygalacturonase, sucrase and β-galactosidase activities. The active fractions were pooled and lyophilized.

Determination of pH and Thermal Stability of Enzyme Preparation

All experiments were conducted using the Biogel-P100 eluted active fraction as source of partially purified enzyme. The pH optimum of polygalacturnase was determined in the pH range 2.0 – 9.0. The incubation mixture (2 mL) containing 4.0 U of enzyme was pre-incubated with 0.1M buffers of various 37ºC for 10 min. The reaction started with the addition of substrate (1 %; w/v pectin). Residual enzyme activity was determined as mentioned later. The pH stability of enzyme was determined by incubating a mixture containing 10 U/ mL of plant polygalacturonase at 37ºC for 1 h at different pH buffers (pH 2.0 – 9.0). Residual enzyme activity was determined after dilution of the enzyme in optimum pH buffer (0.1M glycine-HCl buffer, pH 3.0). Same experimental methodology was followed for determination of pH optima and stability of sucrase.

Catalytic potency of polygalacturonase at pH 2.0 was assessed by incubating an enzyme solution (20 U/ mL) in 0.1M glycine-HCl buffer of pH 2.0 at 37°C for 4 h. Aliquots were withdrawn at different time intervals and the residual enzyme activities were determined at optimal buffer.

The optimum temperature for polygalacturonase was determined in the temperature range from 30 – 80 ^{0}C. In the reaction mixture (2 mL), 0.5U of enzyme was incubated with 1% (w/v) pectin in 0.1 M glycine-HCl buffer, pH 3.0 at different temperatures for 15 min. Temperature stability was determined in the range of 30-80 ºC. The incubation mixtures containing 10 U/ mL of enzyme in requisite buffer solutions were pre-incubated at different temperatures for 30 min. Fixed aliquots were withdrawn and residual enzyme activities determined. Same experimental methodology was undertaken for determination of temperature optima and stability of sucrase.

Optimal pH for β-galactosidase was determined in the pH range 2.0 – 9.0. The incubation mixture (0.2 mL) containing 0.4 U

of enzyme was pre-incubated with 0.1M buffers of various 37 ºC for 10 min. The reaction started with the addition of substrate (4 mg/ mL ONP-Gal). Residual enzyme activity was determined as mentioned later. pH stability, temperature optima and temperature stability were determined accordingly.

Effect of Various Metal Ions on Polygalacturonase, Sucrase and β-Galactosidase

In the reaction mixture (2 ml), 0.5U of polygalacturonase in 0.1M glycine-HCl buffer, pH 3.0 was pre-incubated for 15 min at 37ºC in presence of different metal ions (2mM). Reaction was initiated with the addition of 1% (w/v) substrate and the incubation continued for 15 min at 50°C. The residual amylase enzyme activities were subsequently determined. 'Control' sets containing same amount of enzyme in absence metal ions were run parallel. The same experimental procedure was followed using similar amount of plant sucrase. Effect of various metal plant β-galactosidase was determined as follows: In the reaction mixture (200 µl), 0.1U of β-galactosidase in 0.1M glycine-HCl buffer, pH 3.0 was pre-incubated for 15 min at 37ºC in presence of different metal ions (2mM). Reaction was initiated with the addition of 1% (w/v) substrate (ONPG) and the incubation continued for 20 min at 45 ^{0}C. Reaction was terminated by addition of 0.8 mL of 1M sodium carbonate solution. Residual enzyme activity was determined as mentioned later.

Substrate Specificity

Activity of the plant enzyme for the liberation of reducing groups from various substrates [purified pectin, crude guava pectin, crude apple pectin, crude cirrus pectin, xylan, soluble starch, amylopectin, glycogen (oyster shell), carboxymethyl cellulose, dextran, sucrose, inulin, raffinose, melizitose, levan and agar] was determined at 0.1M acetate buffer, pH 5.0 at 45 ^{0}C. Effect of the plant enzyme on disaccharides like maltose, isomaltose, and lactose was also determined by measuring the amount of glucose liberated from the substrates.

Determination of K_m and V_{max}

Enzyme (polygalacturonase, sucrase and β-galactosidase) activities at their optimal pH and temperature conditions were determined (as mentioned under analytical methods) using a substrate concentration range of 1-20 mg / mL. K_m and V_{max} values were determined by Lineweaver-Burk plot followed by regression analysis to determine the slope of the best fitting line.

Effect of Various Amino Acid Modifying Agents on Enzyme Cocktail

100 mM stock solution of various amino acid modifying agents (Iodoacetic acid, iodoacetamide, DTNB, PCMB, water soluble carbodiimide) were prepared by dissolving each of them in their recommended **[13]** pH buffer. In incubation mixtures (1 mL), 20 U of polygalacturonase was pre-incubated separately with 10 mM of the above mentioned chemicals (EDAC used was 50 mM) at 37°C for 3 h. Fixed aliquots were withdrawn from each of the above samples, diluted with optimal buffer, and residual enzyme activity was determined. Inhibition kinetics with time was studied. Same experiments were repeated using similar amount of sucrase.

Hydrolysis of Crude Pectin From Various Sources by Plant Polygalacturonase

Guava cake (peel, pulp and seeds) was obtained directly from a commercial guava juice processing plant. The guava cake (100 g) was washed twice with water, wrapped in cheesecloth and pressed to remove liquid and dried in a hot air oven at 55 ^{0}C for 10 h to obtain dried press cake. The dried sample was ground using a warring blender and immediately packed in an air tight container and stored at 4°C. Pectin was extracted from guava cake powder by the method of Iglesias and Lozano (2004) with a slight modification **[14]**. The powder was washed with hot water (65°C) for 15 min and then filtered through a muslin cloth. The pH of slurry (100 mL) was adjusted to 3.0 - 3.5 with 4 N hydrochloric acid and the sample was kept in a water bath at 75°C for 2 h with periodic stirring. The solution was filtered through a Buchner funnel. To the filtrate (brought to 4°C), 4 vol. of acidified ethanol (1 N HCl: 95% ethanol = 1:3) was added and the sample was kept overnight at 4°C. The sample was centrifuged; the precipitate obtained was washed thrice with 70% ethanol and finally air dried to obtain dry powder. The dry powder was used as a source of crude pectin. Total carbohydrate content of the powder was determined using anthrone reagent **[15]**. The same methodology was undertaken to obtain crude pectin from apple and orange peel.

Hydrolysis of crude pectin from various sources was determined as follows: In a reaction mixture (25 mL), 2 % (w/v) of crude pectin (from various sources) in 0.1 M glycine-HCl buffer was incubated with 20 U/ mL of plant polygalacturonase. Fixed aliquots were withdrawn at different time intervals and the amount of reducing sugar formed was estimated as mentioned later (under analytical procedures). % hydrolysis was calculated using the formula:

Hydrolysis (%) = [total reducing sugar (galacturonic acid equivalent)/ total carbohydrate] X 100

Hydrolysis and Time Kinetics of Sucrose, Raffinose, Inulin and Levan

In four different reaction mixtures (25 mL), 2 % (w/v) each of sucrose, raffinose, inulin, and levan in 0.1 M glycine-HCl buffer, pH 3.0 was incubated separately with 20 U/ mL of plant sucrase. Fixed aliquots were withdrawn at different time intervals and the amount of reducing sugar formed was estimated as mentioned later (under analytical procedures). Total glucose formed was determined using GOD-POD reagent (under analytical procedures). % hydrolysis was calculated using the formula: Hydrolysis (%) = [total reducing sugar (glucose equivalent)/ total carbohydrate] X 100.

Analytical Procedures

Plant polygalturonase (pectinase) activity was determined in 0.1M glycine-HCl buffer, pH 3.0 at 50 ^{0}C using 1 % (w/v) pectin as substrate. Reducing sugar formed was estimated by dinitro salicylic acid reagent (DNSA) according to Sengupta *et al*., **[16]**. Sucrase, amylase, xylanase, and dextranase activities were determined similarly in 0.1M acetate buffer, pH 4.0 using 1 % (w/v) of sucrose, starch, xylan and dextran respectively. Pullulanse, 1-O-methyl-α-D-glucosidase, raffinase, melizitase and inulinase activities were also determined in the same way using 1 % (w/v) each of pullulan, 1-O-methyl-α-D-glucoside, raffinose, melizitose and inulin respectively as substrate. Pectin lyase activity was determined in the following way. Reaction mixture (1 mL) containing 0.5 % (w/v) citrus pectin in 0.1 M acetate buffer, pH 5.0 was incubated with 0.1 mL of plant enzyme for 60 min at 45 ^{0}C. Pectin lyase activity was determined by measuring the increase in absorbance of the enzymatic products at 235 nm at regular time interval.

Maltase, isomaltase, maltotriose and maltotetraose hydrolyzing activities were determined at pH 5.0 at 45 ^{0}C using 0.5 % (w/v) each of maltose, isomaltose, maltotriose, and maltotetraose as substrates. Total glucose formed was estimated by glucose oxidase–peroxidase (GOD-POD) reagent as described by Bergmeyer *et al.*, **[17]**. Enzyme activity (U) was expressed in terms of μ mole of glucose liberated per minute under the assay conditions. β-glucosidase activity was determined in 0.1M glycine-HCl buffer, pH 3.0 at 45°C using o-nitrophenyl- β-D-galactopyranoside (4mg/mL) as substrate. The amount of o-nitro-phenol released (ONP) under the assay conditions was measured at 410 nm. Enzyme

activity (U) was expressed in terms of μ mole of ONP liberated per minute under the assay conditions. Protein was estimated using coomassie blue assay reagent **[18]**.

RESULTS

Preparation and Storage Stability of Pectinase and Sucrase from *C. gigantea*

*C. gigantea a*s a rich source of extracellular polygalacturonase and sucrase was first reported by Mukherjee **[19]**. The storage stability of enzyme in *C. gigantea* latex and chopped stem-bark was appreciably good. The latex (liquid) remained stable at 4 ^{0}C, retaining about 78-80 % enzyme activities till 6 months of study **(Figure 1)**. Chopped stem-bark when lyophilized and kept at room temperature in sealed containers, retained more or less full enzyme activity over 6 months of storage **(Figure 1)**. In the process for the preparation of commercial enzyme grades from the plant, enzyme extraction protocol from plant stem-bark was optimized. The extraction buffer selected was 0.1M acetate buffer, pH 5.0. Enzyme extracted in the above aqueous medium remained stable in cold (4°C) till six months of study **(Figure 1)**. Substantial amount of enzyme was simply leached out from the cut stem-bark chips when stirred in the buffer, preferably at 45°C **(Table 1)**. Bark and stick of the plant when extracted separately showed that almost 90 % of enzyme activity was present in stem-bark **(Table 1)**.

However, maximum yield was obtained from the latex, collected from stem node, leaves and fruits. Ratio of pectinase: sucrase: β-galactosidase was found to be 340 $\pm$ 20: 65 $\pm$ 3: 6 $\pm$ 1 U/ mL of latex respectively and 170000 $\pm$ 5000: 35000 $\pm$ 2500: 3000 $\pm$.100 U/ 100 g of dry latex powder **(Table 1)**. However, the latex showed negligible pectin lyase activity (data not given). Lyophilized ammonium sulphate precipitated latex protein contained 15000 $\pm$ 200 U of pectinase / g of solid **(Table 1)** and the preparation retained about 92 % of its original activities till 6 months of study conducted when stored at 0-4 ^{0}C **(Figure 1)**.

With the above extraction protocol, recovery of (U/ 100 g of fresh tissue) pectinase, sucrase and β-galactosidase from stem-bark was 11500 $\pm$ 500, 975 $\pm$ 20, 1000 $\pm$ 100 respectively (Table 1). Ultra-filtered, lyophilized stem-bark extract contained 20000 $\pm$ 150 and 3700 $\pm$ 100 U of pectinase and sucrase respectively / 100 g of dry solid (Table 1). The preparation was stable upto 6 months of study conducted when stored at 4 ^{0}C, retainging about 80 5 of its original activity. (Fig.1). Freshly collected and chopped stem-bark pieces, lyophilized to dryness and subsequently ground into fine

powder gave a crude enzyme preparation containing (U/ 100 g of dry powder) 31300 ± 1000 and 2785 ± 150 of pectinase and sucrase respectively **(Table 1)**. However, fresh stem-bark stored at 0 ^{0}C lost about 40 % of enzyme activity during 6 months of storage **(Figure 1)**.

Partial Purification of Polygalacturonase, Sucrase and β-Galactosidase

Carbohydrases from the latex of *C. gigantea* were partially purified by ammoinum sulphate precipitation and gel filtration chromatography. **Table 2** indicates that ammonium sulphate precipitation (80 % saturation) recovered about 90 and 85 % of polygalacturonase and sucrase activities respectively. Gel filtration chromatography (Biogel P-100) recovered about 73 % and 64% of polygalacturonase and sucrase activities respectively. Gel filtration chromatography resulted in two major protein peaks: fraction 15-22 and fraction 27-38 **(Figure 2)**. The former protein peak contained all the enzyme activities (polygalacturonase, sucrase and β-galactosidase). The above method resulted in 2.94 (almost 3 free fold) and 2.54 fold purification of polygalacturonase and sucrase respectively.

Physicochemical Properties of *Calotropis gigantea* Enzyme Extract

Figure 3 (a) shows the pH optima of different enzymes from *C. gigantea*. The enzyme polygalacturonase showed optimal activity at pH 3 – 4, retaining about 87 ± 2 % and 84 ± 2 % activity at pH 2.0 and 7.0 respectively. However the enzyme could retain only 18 ± 2 % activity at pH 8.0. *C. gigantea* sucrase exhibited its optimal activity at pH 4-5, retaining about 77 ± 2 % and 60 ± 2 % activity at pH 2.0 and 7.0 respectively. Sucrase retained about 25 ± 1 % activity at pH 8.0. β-galactosidase was optimally active at pH 3.0 with about 55 ± 4 % and 8 ± 2 % activity at pH 2.0 and 7.0 respectively.

Figure 3 (b) shows the pH stability of the carbohydrases from *C. gigantea*. Sucrase showed a remarkable stability in the pH range of 2-8. Polygalacturonase was stable in the pH range of 2-7 retaining about 95 % and 75 % activity at pH 2 and 7 respectively.

β-galactosidase was stable in the pH range of 3-5, retaining about 55% and 60 % activity at pH 2 and 6 respectively.

The inactivation kinetics at pH 2 **(Figure 4)**, determined for *C. gigantea* polygalacturonase showed that the enzyme retained more than 70 % activity over a period of 3 h.

C. gigantea polygalacturonase displayed optimal activity at 50°C retaining about 85 % and 70 % of activity at 60 and 70°C respectively (data not shown). The enzyme

was stable upto a temperature of 50°C, but activity fell sharply beyond 55°C (data not shown). Sucrase and β-galactosidase were optimally active at 45°C, retaining about 75 % and 62 % of enzyme activity at 60°C and 70°C respectively (data not shown). Both the enzymes (sucrase and β-galactosidase) were found to be stable upto a temperature of 50°C. Polygalacturonase activity was not affected in presence of (2 mM) heavy metals like Cd^{2+}, Pb^{2+}, Cu^{2+}, and Ag^{+}. However, Hg^{2+} at the same concentration inhibited about 50 % of enzyme activity **(Table 3)**. The enzyme activity was also strongly inhibited (about 35 %) in presence of 1mM Hg^{2+}. Ca^{2+}, at a concentration of 2-10 mM increased the enzyme activity but inhibited the same (by about 4 – 5 %) at a concentration of 20 mM. The enzyme activity was increased by about 18 % in presence of 2 mM Zn^{2+}. Sucrase was strongly affected in presence of heavy metals like Pb^{2+}, Ag^{2+}, and Hg^{2+}. Activity was completely lost in presence of Ag^{+} and Hg^{2+} **(Table 3)**. Zn^{2+}, Mg^{2+}, and Fe^{3+} slightly enhanced the enzyme activity while Ca^{2+} showed no effect. Both polygalacturonase and sucrase were not affected in presence of 1-20 mM EDTA (data not shown). β-galactosidase activity was almost completely inhibited in presence of 1mM Hg^{2+} **(Table 3)**. Zn^{2+}, Mg^{2+}, and Ca^{2+} caused slight improvement of enzyme activity.

Figure 5 shows the effect of various amino acid modifying agents on *C. gigantea* polygalacturonase and sucrase. Polygalacturonase activity was slightly affected in presence of 10 mM DTNB **(Figure 5)**. Iodoacetic acid and iodoacetamide did not inhibit polygalacturonase activity. The enzyme retained about 90 % of its activity in presence of 50 mM EDAC. However, 10 mM PCMB strongly inhibited enzyme polygalacturonase activity, inhibiting about 50 % of enzyme activity in 3 h **(Figure 5)**. The enzyme sucrase was strongly inhibited by PCMB, inhibiting about 80 % of enzyme activity within 30 min. The enzyme however remained insensitive to the presence of iodoacetamide and iodoacetic acid. EDAC and DTNB slightly inhibited sucrase activity **(Figure 5)**.

Substrate Specificity and Kinetics of Pectin Hydrolysis

Table 4 describes enzyme activities of the crude aqueous extract of *C. gigantea*. The enzyme cocktail from was most active on pectin. However, it displayed slight pectin lyase activity (data not shown). It appears from the table that the preparation had substantial amount of sucrose, raffinose and

melezitose hydrolyzing activities. The enzyme cocktail also showed maltase, isomaltase activities. The preparation showed slight amylase, xylanase and dextranase activities **(Table 4)**. However, no pullulanase activity was detected though it showed slight levan hydrolyzing activity.

C. gigantea polygalacuronase readily hydrolyzed crude guava, apple and citrus peel pectin. The enzyme hydrolyzed about 80 % of pure pectin in about 4 h, while it hydrolyzed about 75 % of guava pectin in same time. The enzyme hydrolyzed 70 % of citrus pectin in 4 h while it could hydrolyze only 64 % of apple pectin in the same time **(Figure 6)**. The reaction entered plateau phase by about 210 min. The enzyme cocktail also hydrolyzed sucrose and raffinose (Fig.7). Total glucose produced from sucrose hydrolysis was about 220 $\pm$ 10 mg (data not shown). However, the enzyme poorly hydrolyzed inulin (12 %) and levan (14 %).

K_m and V_{max} values obtained for *C.gigantea* polygalacturonase using pectin as substrate were 3.4.mg/ mL and 1.096 µmole/ min respectively. K_m and V_{max} values obtained for sucrase were 2.8 mg/mL (sucrose as substrate) and 0.65 µmole / min respectively, while those obtained for β-galactosidase were 1.2 mg/mL (PNPG-β-gal as substrate) and 0.086 µmole / min respectively.

DISCUSSION

Pectinases are usually produced by microorganisms, the filamentous fungi being the most common choice for commercial production. Microbial production of pectinases has been extensively studied **[20, 21]**. Pectinase have been reported from *Aspergillus flavus* **[22]**; *Aspergillus sp.* **[23]**; *Penicilluim italicum* **[24]**; *Penicillium roqueforti* **[25]**; *Penicillium expansum* **[26]** and pectolytic moulds **[27]**. The largest industrial application of pectinase is in fruit juice extraction and clarification. A mixture of pectinases and amylases is used to clarify fruit juices and can decrease filtration time up to 50% **[28]**. Treatment of fruit pulps with pectinases also showed an increase in fruit juice volume from banana, grapes and apples **[29]**. Pectinases in combination with cellulases, arabinases and xylanases, have been used to increase the pressing efficiency of the fruits for juice extraction **[30]**. A process for the preparation of an enzyme composition containing a mixture of pectinase and xylanase (from *Termitomyces clypeatus*) useful for clarification of non-citrus fruit juice was developed **[31]**. However, the process was not suitable for citrus fruit juice clarification because of the low pH of citrus fruit juice. Thus, isolation and characterization of acid stable pectinase

capable of hydrolyzing both citrus and non-citrus fruit pectin is essentially required.

Over-production of pectinolytic enzymes in plants is very rare. This is the first report on overproduction of pectinolytic enzymes in plant tissue and latex. *Calotropis gigantea* is a well known medicinal plant of India and has been used in Ayurvedic system of medicine since ancient times. A thiol protease (procerain) was purified and characterized from the latex of *Calotropis procera*. The 28.8 KDa enzyme protein was optimally active at a pH range of 7 - 9 and at a temperature range of 55 - 60°C **[32]**. However, no carbohydrases from *C. gigantea* have been reported till date.

C.gigantea appeared to be a rich source of extracellular polygalacturonase and sucrase-the yield being 170000 $\pm$ 5000 and 35000 $\pm$ 2500 U/ 100 g of latex, respectively and 34000 $\pm$ 2000 and 6500 $\pm$ 300 U/ 100 mL of fresh latex. It must be mentioned that solid state fermentation of *Aspergillus niger* URM4645 on forage palm yielded 6619 U of polygalacturonase / 100 g of substrate at 96 h of fermentation **[33]**. Freitas *et al*. (2006) reported that solid state fermentation of *Aspergillus* sp. using wheat bran with pectin yielded 190 U / 100 mL of polygalacturonase activity at 72 h of fermentation **[34]**. Fontana *et al*. (2005) reported that solid state fermentation of *Aspergillus* sp., using wheat bran and citric pectin substrates, yielded 15200 U of polygalacturonase/ 100 g of substrate at 72 h of fermentation **[35]**.

New enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have always been the focus of research. Production of pectinolytic and glycosidases from microbial sources requires maintained upstream process technology, which is cost-effective. No such upstream technology is required for extraction of plant enzymes. The plant source itself acts like a bioreactor producing the enzyme, which needs only to be harvested and extracted. Plant latex appears to be a cheap and source of commercial enzymes, as collecting the latex does not harm the plant or hamper the plant growth in any way. Moreover, latex can be collected at regular intervals from various parts of the plant (stem-node, leaves, and fruits). It must be mentioned that plant latex has been reported be a source of several proteolytic enzymes like papian, ficin **[36]**, procerain **[32]**.

In the process for the preparation of commercial enzyme grades from the plant, enzyme extraction protocol from plant stem-bark and latex was optimized. Since the plant (*C. gigantea*) contains gum, extraction of

enzyme from stem was low at ambient temperature but gradually became high with the increase in temperature upto 45°C. Leaching of enzymes from chopped stem-bark was also high at 45°C. *C. gigantea* polygalacturonase was found to be extracellular in origin with the bulk activity present in the latex **(Table 1)**. The stem-bark also contained appreciable amounts of enzyme. Various preparations of *C.gigantea* polygalacturonase **(Table 1)** with high potency were prepared. Chopped plant stem bark pieces dried by lyophilization and kept at room temperature in sealed containers retained more or less full enzyme activity over 6 months of storage **(Figure 1)**. The lyophilized stem-bark extract also showed excellent stability **(Figure 1)**. It is to be noted that the ratio of polygalacturonase: sucrase was higher in stem-bark compared to latex **(Table 1)**. The stem-bark gave a poor yield of sucrase but a better yield of β-galactosidase (data not shown). Excellent recovery of polygalacturonase and sucrase was achieved by ammonium sulphate precipitation of the crude extract **(Table 1)**.

Though polygalacturonase has been reported from various plants and fruits but none of them are obtained in amount suitable for commercial use. Role of polygalacturonase in fruit ripening has also been much studied. Plant peel could be a potential source of pectinase. Recently, pectinase (polygalacturonase) was extracted and purified from mango (*Mangifera indica* cv. Chokanan) peel using the aqueous two-phase system. The 31 KDa enzyme protein was optimally active at 60ºC **[37]**. However, the enzyme was optimally active at pH 8.0 and was stable in the pH range 5 to 9 thus making it unsuitable for clarification of citrus fruit juice. However, *C.gigantea* polygalacturonase was stable in the acid pH range **(Figure 3)** and retained more than 70 % of its original activity over a period of 3 h when incubated in buffer of pH 2 **(Figure 4)**. *C.gigantea* polygalacturonase activity increased (about 12 %) in presence of Ca^{2+} (see Table) similar to that of pectinase isolated from mango peel **[37]**. An endopolygalacturonase (EC 3.2.1.151) was also purified from ripening tomato fruits, with a native molecular weight of about 11 5 KDa **[38]**. Thiol polygalacturonase isoenzyme was purified to homogeneity from banana fruit pulp. The 90 $\pm$ 10 KDa with a subunit molecular weight of 29 $\pm$ 2 KDa enzyme protein was inhibited by PCMB **[39]**.

Besides pectin, the *C. gigantea* enzyme extract was also capable of hydrolyzing polysaccharides like starch, xylan, and dextran. Moreover, *C. gigantea*

polygalacturonase could readily hydrolyze crude pectin from apple, guava and citrus peel appreciably **(Figure 6)**. Thus, polygalacturonase accompanied with amylase and xylanase activities make the plant enzyme an excellent commercial choice for both citrus and non-citrus fruit juice clarification. *C.gigantea* polygalacturonase was not highly sensitive to heavy metals and thiol blocking agents like DTNB, PCMB, iodoacetic acid and iodoacetamide did not inhibits its activity **(Figure 5)** thus suggesting it to be a non-thiol enzyme protein, where the SH group (s) is not directly involved in the catalytic activity of the enzyme.

The enzyme cocktail was also rich in sucrase and hydrolyzed trisaccharides like raffinose and melezitose, and maltotriose. However, it showed poor activity on inulin and levan **(Figure 6)**. The plant latex also appeared to be a source of maltase and isomaltase active in acidic pH **(Table 4)**. Invertase activity is present in many plant tissues including seedlings of beans **[40]**, lentils **[41]**, peas **[42]**, and other species. Plant invertases (sucrase) have been classified as vacuolar, extracellular (cell wall), and cytosolic **[42]**. Plant invertases are also classified as acid or alkaline invertases on the basis of their pH optima **[43]**. Both types of enzymes often occur in the same tissue; the acid invertase is usually associated with the cell wall whereas the alkaline invertase is located in the cytoplasm **[44]**. Acid invertases have been purified from several plant species. Activity of acid invertases is usually inhibited by heavy metal ions such as Hg^{2+} and Ag^{+} **[42]**. We recently, reported the presence of acid sucrase activity in the stem extract of a non-cereal, non-leguminous plant, *Tinospora cordifolia* **[5]**. The plant *(Tinospora cordifolia*) contained 45 $\pm$ 5 U of sucrase/ g of fresh stem; the yield being lower compared to sucrase from *C.gigantea* latex (65 $\pm$ 3 U/ mL of fresh latex). *T. cordifolia* sucrase was also active on raffinose and melizitose **[5]** similar to that of *C.gigantea* sucrase.

C.gigantea sucrase is an acid sucrase optimally active in the pH range of 3-5 and remarkably stable in the pH range of 2-8 **(Figure 3)**. The enzyme was not affected by thiol alkylating agents like iodoacetic acid and iodoacentamide **(Figure 3)** similar to that of oat invertase **[45]**. Hg^{2+} and Ag^{+} strongly inhibited *C. gigantea* sucrase similar to that of oat **[44]** and other acid invertases **[42]**.

CONCLUSION

All the above observations strongly suggest that *C. gigantea* might be a potential commercial source of plant polygalacturonase useful for both non-citrus and citrus fruit juice clarification. Further studies on purification of

the enzymes will throw more light on their mode of action and role in the plant physiology.

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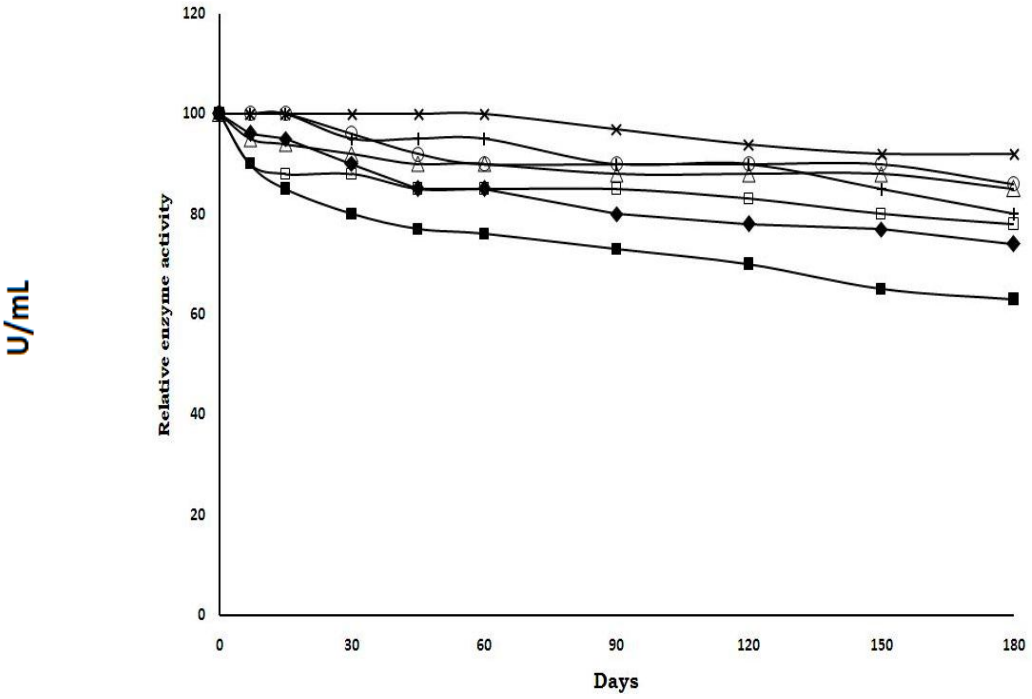


Figure 1: Storage Stability of Raw Material

NOTE: The plant material stored in different ways (as mentioned in the text) was assessed time to time for the retention of polygalacturonase and sucrase activities till 6 months; Fresh latex stored at 0°C, Dry latex powder stored at 0°C; Fresh stem-bark extract stored at 0-4°C; Fresh stem-bark stored at 0°C; Lyophilized (dry) stem-bark stored at room temperature; Ammonium sulphate precipitated dialyzed latex protein stored at 0-4°C; Lyophilized (dry) stem-bark stored at 0-4°C

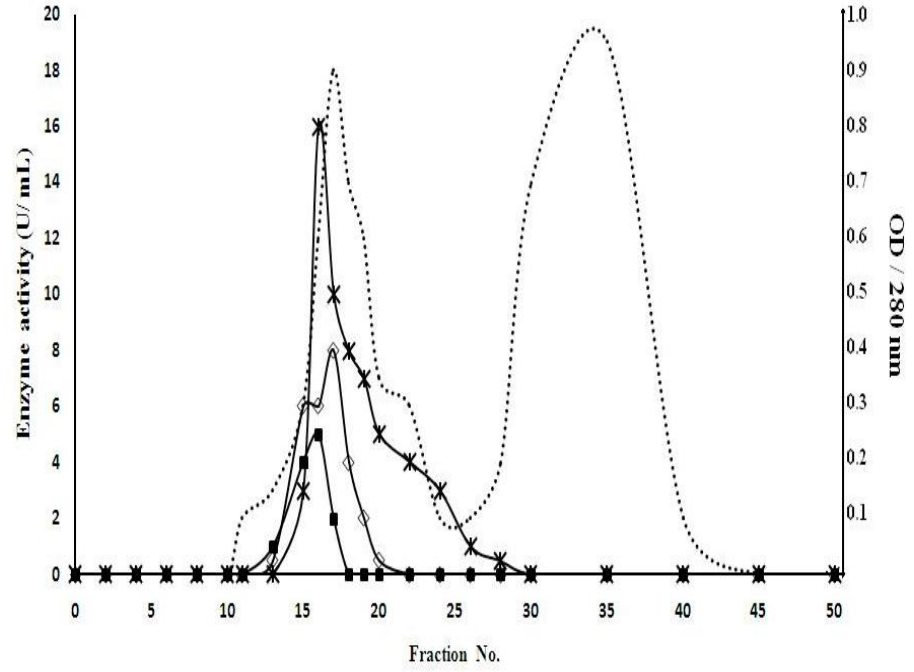
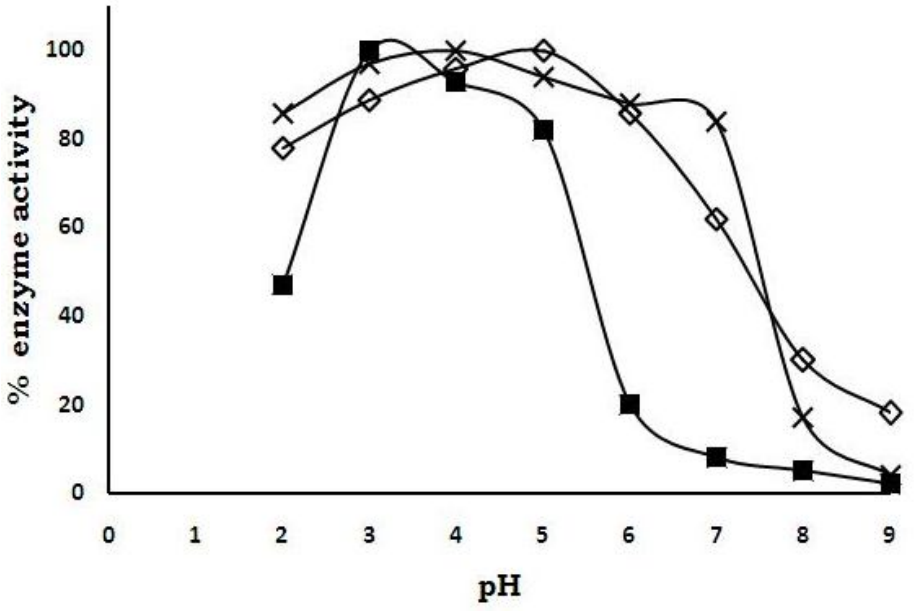


Figure 2: Partial Purification of *C. gigantea* Carbohydrases

NOTE: Experimental details have been mentioned in the text; polygalacturonase; β-galactosidase; sucrase



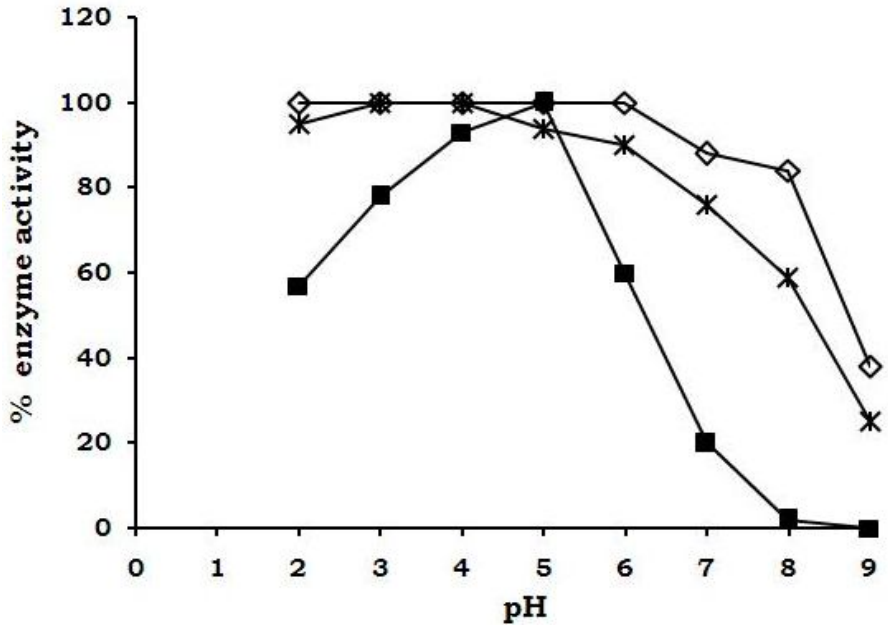


Figure 3: pH Optima and Stability of *C. gigantea* Carbohydrases
NOTE: Experimental details have been mentioned in the text. The data represent the average value of triplicate similar sets of experiments; ✳ polygalacturonase; ■ β-galactosidase; ◈ sucrose

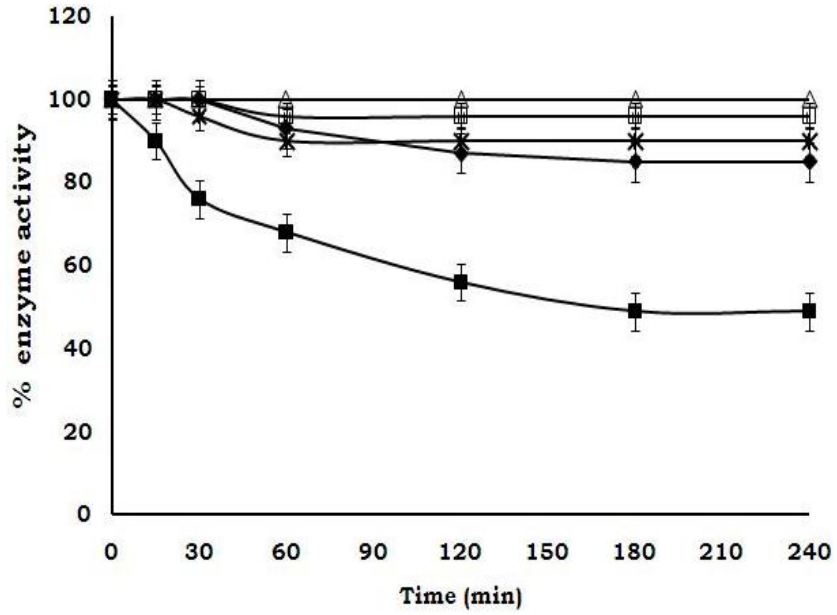


Figure 4: Assessment of Catalytic Potency of *C.gigantea* polygalacturonase in pH 2 Experimental Details Have Been Mentioned in the Text. The Data Represent the Average Value Of Triplicate Similar Sets of Experiments

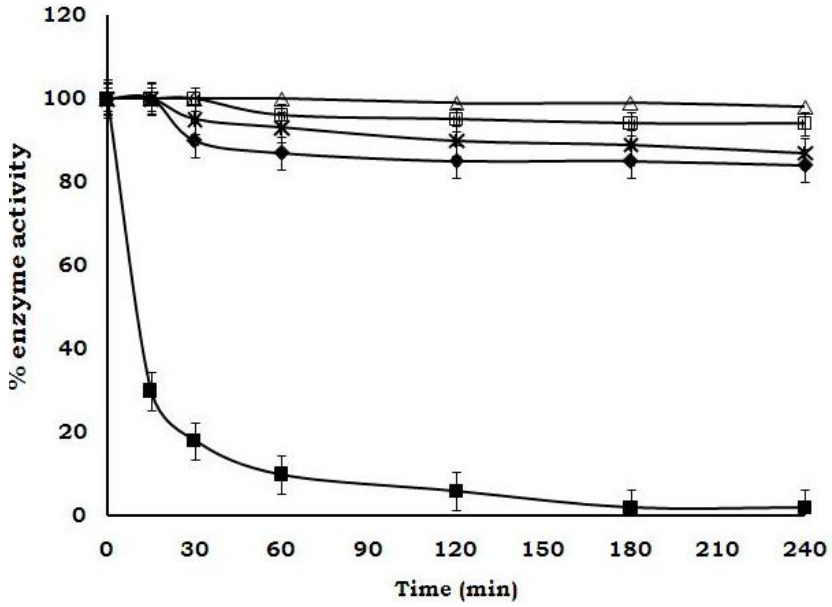
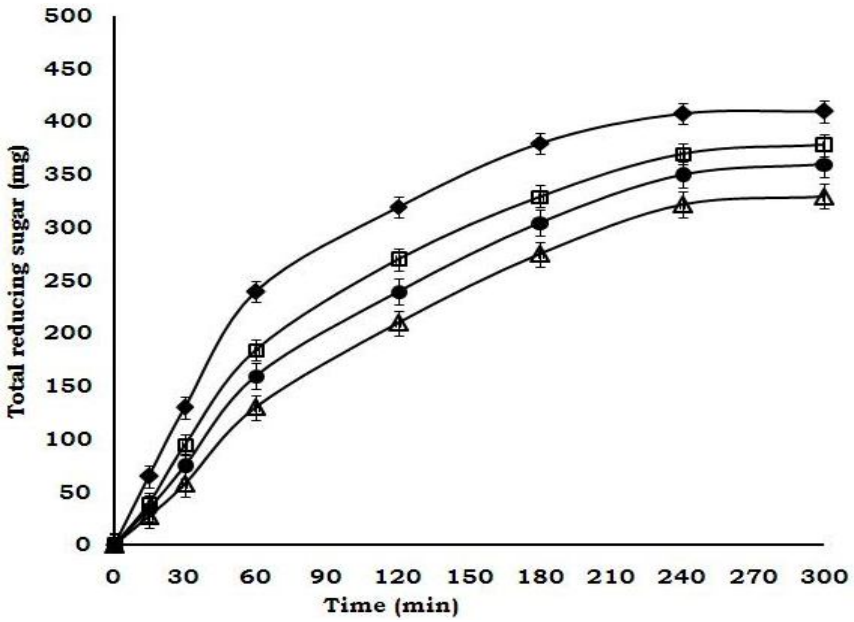


Figure 5: Effect of Various Amino Acid Modifying Agents on *C. gigantea* Polygalacturonase and Sucrase

NOTE: In incubation mixtures (1mL), 20U of enzyme was pre-incubated separately with 10 mM of the above mentioned chemicals (EDAC used was 50 mM) at 37°C for 3 hours. Fixed aliquots were withdrawn from each of the above samples, diluted with optimal buffer, and residual enzyme activity was determined; Data represent the average values of similar triplicate sets of experiments with ± SD; DTNB (10 mM); PCMB (10 mM); Iodoacetic acid (10 mM); iodoacetamide (10 mM); EDAC (50mM)

(a)



(b)

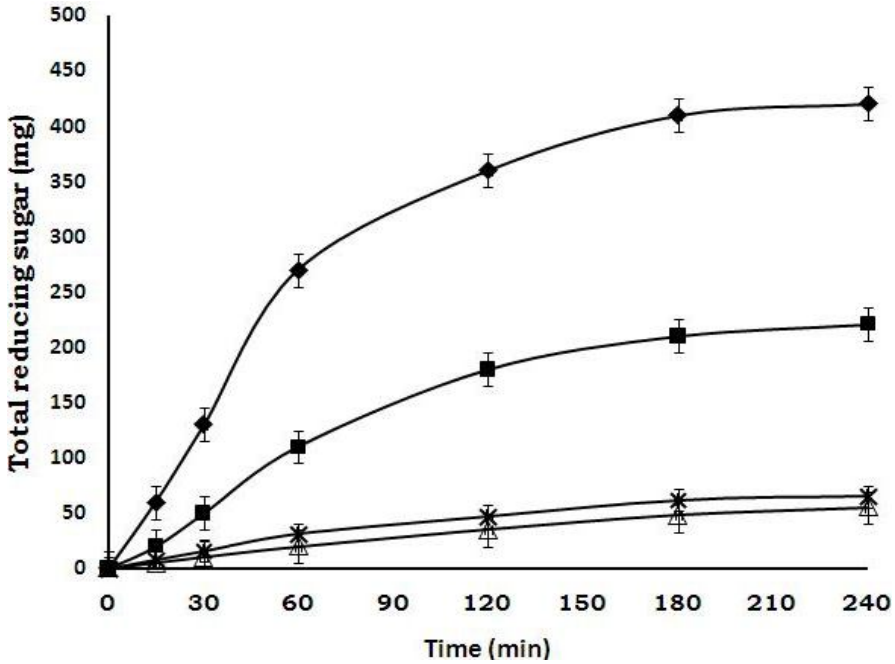


Figure 6: Kinetic Study of Hydrolysis of Various Substrates by *C. gigantea* Carbohydrases

(a) Hydrolysis of purified and crude pectin from various sources (guava, apple, citrus peel) by the partially purified *C.gigantea* polygalacturonase. Crude pectin was isolated from their sources as mentioned in the text. Experimental details have been mentioned in the text. The data represent the average of triplicate similar sets of experiments with ± SD; ◆ purified pectin; □ crude guava pectin; ● crude citrus pectin; △ crude apple pectin

(b) Hydrolysis of sucrose, raffionose, inulin. and levan by the partially purified *C.gigantea* sucrase. Experimental details have been mentioned in the text. The data represent the average of triplicate similar sets of experiments with ± SD; ◆ sucrose; ■ raffinose; △ inulin; ✱ levan

Table 1: Distribution of Pectinase and Suscrase in Plant and Relation of Enzyme Yield with Various Extraction Methods

		Enzyme recovery	
	Method of extraction	**Pectinase**	**Sucrase**
Stem-bark	**a) Leaching (chopped stem-bark) at (i) 4 ^{0}C**	**3100 ± 300**	**27 ± 3**
	(ii) 50^{0}C	**6000 ± 400 (U/ 100 g fresh tissue)**	**58 ± 2 (U/ 100 g fresh tissue)**
	b) Extraction of blended mass at (i) Room temperature	**9500 ± 300 (U/100 g fresh tissue)**	**ND**
	(ii) 45 ^{0}C	**11500 ± 500 (U/ 100 g of fresh tissue)**	**975 ± 20 (U/ 100 g of fresh tissue)**
	(iii) Extraction of blended mass at 50^{0}C followed by ultrafiltration (PM-10) and lyophilization	**2000000 ± 15000 (U/ 100 g of dry powder)**	**370000±10000 (U/ 100 g of dry powder)**
	c) Lyophilization of fresh stem-bark pieces	**31300±1000 (U/ 100 g dry weight)**	**2785 ± 150 (U/ 100 g dry weight)**
Whole stem	**Extraction of blended mass at 50 ^{0}C**	**4400 ± 50 (U/ 100 g fresh stem)**	**570 ± 50 (U/ 100 g fresh stem)**
Latex	**Liquid**	**34000 ± 2000 (U/ 100 mL of latex)**	**6500 ± 300 (U/ 100 mL of latex)**
	Dry latex powder	**170000 ± 5000 (U/ 100 g dry latex)**	**35000 ± 2500 (U/ 100 g of dry latex)**
	Lyophilized ammonium sulphate precipitated, dialyzed, latex protein	**1500000 ± 20000 (U/ 100 g of dry solid)**	**2,80,000± 12,000 (U/ 100 g of dry solid)**

Table 2: Partial Purification of *Calotropis gigantea* Carbohydrases

Polygalacturonase					
Sample	**Total enzyme activity (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Fold purification**	**% Recovery**
Crude Latex (20 mL)	**7200**	**400**	**18.00**	**1.00**	**100**
$(NH_4)_2SO4$ precipitate (5mL)	**6500**	**310**	**20.96**	**1.164**	**90.2**
Biogel P-100 (2 mL)	**5300**	**100**	**53.0**	**2.94**	**73.6**
Sucrase					
Crude Latex (20 mL)	**1400**	**400**	**3.5**	**1.00**	**100**
$(NH_4)_2SO4$ precipitate (5 mL)	**1200**	**310**	**3.8**	**1.08**	**85.7**
Biogel P-100 (2 mL)	**900**	**100**	**9.0**	**2.57**	**64.2**

Table 3: Effect of various cations on *C. gigantea* polygalacturonase and sucrase activities; Reaction mixtures (2 ml), containing 0.5U of polygalacturonase and sucrase separately in 0.1M glycine-HCl buffer, pH 3.0 were pre-incubated for 15 min at 37ºC in presence of different metal ions (2mM). Reaction was initiated with the addition of 1% (w/v) substrate and the incubation continued for 15 min at 45 ^{0}C. β-galactosidase assay methodology has been mentioned in the text. Enzyme activity determined in absence of metal ion (control) was marked as 100. Data expressed here are the average of triplicate similar sets of experiments

Metal ions (2mM)	Pectinase	Sucrase	β-galactosidase
Control	**100**	**100**	**100**
Pb^{2+}	**100**	**49**	**94**
Cd^{2+}	**100**	**100**	**95**
Ag^{+}	**99**	**0**	**98**
Hg^{2+}	**51**	**0**	**0**
1mM	**35**	**0**	**5**
Cu^{2+}	**96**	**91**	**94**
Zn^{2+}	**118**	**104**	**105**
Mg^{2+}	**105**	**104**	**106**
Fe^{3+}	**90**	**108**	**98**
Ca^{2+}	**105**	**100**	**107**
4mM	**108**	**100**	**107**
10mM	**112**	**100**	**102**
20mM	**96**	**-**	**-**

Table 4: Substrate Specificity of *Calotropis gigantea* Enzyme Extract

Substrate (1 % ; w/v)	Specific activity (U /mg protein)
Pectin (Pure, SRL)	53 ± 4.2
Crude pectin (guava)	50 ± 2.5
Crude pectin (apple)	36 ± 3.0
Crude pectin (citrus peel)	48 ± 1.5
Xylan	0.6 ± 0.05
Cellulose	Negligible
Starch	0.57 ± 0.01
Dextran	0.54 ± 0.02
Agar	0.85 ± 0.08
Sucrose	11.2 ± 1.5
Maltose (0.5 %w/v)	2.1 ± 0.5
Isomaltose (0.5 %w/v)	1.1 ± 0.2
Maltotriose (0.5 %w/v)	0.9 ± 0.1
ONP-β-Galactoside (4mg/mL)	9.5 ± 0.6
Raffinose	3 ± 0.5
Inulin	0.4 ± 0.05
Levan	0.5 ± 0.04