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Some properties of the polygalacturonase from four Zimbabwean wild fruits (*Uapaca kirkiana*, *Zizphus mauritiana*, *Tamarindus indica* and *Berchemia discolor* fruits)

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Abstract

Polygalacturonase was extracted from ripe *Uapaca kirkiana*, *Zizphus mauritiana*, *Tamarindus indica* and *Berchemia discolor* fruits of Zimbabwe. Protein concentrations and activities of the enzymes in the extracts were determined in the four fruit extracts. The protein concentrations in the enzyme extracts ranged from 0.82 ± 0.17 to 1.97 ± 0.13 mg/ml and enzyme activities from 1.99 ± 0.13 to 6.64 ± 0.38 mmol min^{-1} mg^{-1} in the four fruit extracts. Optimum pH of the enzyme ranged from 4.5 to 5 and optimum temperature from 25 to 37 °C. The enzyme extracts reduced the viscosity of 1% pectin solutions in an experiment which was done together with assay for reducing sugars to prove the activities of the enzyme extracts in the four wild fruits. The K_m and V_{max} ranged from 0.115 to 0.252 mg/ml and 0.0057 to 0.0119 mmol reducing groups/min/mg protein, respectively, in the four polygalacturonase extracts. Calcium chloride and sodium chloride activated the PG from all sources to a greater extend than magnesium chloride and barium chloride. PG from the other three fruits had very little effect on the polysaccharide from *U. kirkiana*.

Keywords: Polygalcturonase; Pectin; Wild fruits; Activities; *Uapaca kirkiana*; *Zizphus mauritiana*; *Tamarindus indica*; *Berchemia discolor*

1. Introduction

Polygalacturonase (PG) falls into the group of enzymes termed polysaccharide lyases or polysaccharide eliminases. Polysaccharide lyases cleave certain activated polysaccharides. PGs are produced by several fruits, which soften on ripening, many fungi and some yeast and bacteria. They have been found in tomatoes (Hobson, 1964, 1965), peaches (McReady & McComb, 1985), avocado and pears (Pomeranz, 1985). PG has been found to play a leading role in fruit ripening. At maturity, fruits undergo many changes, which include the development of colour and aroma and improvements in flavour and texture that make them attractive to a potential consumer (Draper, 1996; Pomeranz, 1985; Skinner, 1995; Tucker & Grieson, 1997).

Softening of fruits is brought about by alterations in the cell wall metabolism and is due frequently to the partial solublisation of pectin or cellulose. Ultrastructural studies on several fruits have demonstrated changes occurring in the cell wall during ripening. In avocado, pear and apple, the middle lamella is changed and the fibrillar structures become sparse during softening (Aggelis, John, & Grierson, 1997; Bateman & Millar, 1966; Berkely, Gooday, & Ellwood, 1990; Brimacombe & Webber, 1994; Hulme, 1993; Kennedy & White, 1993; Pesis, Fuchs, & Zauberman, 1978; Stumpf & Conn, 1997). Tomatoes also show loss of middle lamella structure during ripening (Crookes & Grierson, 1983; Hawthorn, 1985; Underkofler, 1990). These changes during ripening are considered to be a result of the action of cell wall-degrading enzymes (Ben-Arie, Kislev, & Frenkel, 1979; Crookes & Grierson, 1983; Awad & Young, 1990).

Added enzymes are important in food technology because of the roles they play in the composition, processing and shelf life of foods (Crookes & Grierson, 1983; Kilara & Benhura, 1985; Inoue, Nagamutu, & Hatanaka, 1984; Underkofler, 1990). PG is used in the food industry, mainly in the manufacturing of fruit juices (Draper, 1996;

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Hulme, 1993; Linhardt, Galliher, & Cooney, 1986a, 1986b; Voet & Voet, 1992; Whitaker, 1980).

The aim of this work was to extract the PG from four Zimbabwean wild fruits *Zizphus mauritiana*, *Uapaca kirkiana*, *Tamarindus indica* and *Berchemia discolor* and investigate some of the physical properties of the enzyme, such as the enzyme activity, optimum pH, optimum temperature and determine K_m and V_{max}.

The *U. kirkiana* fruit is a fleshy round berry, with a tough reddish brown skin surrounding juicy yellow-brown pulp, in which several hard white-ridged seeds are embedded. It is eaten fresh; the tough seeds and skin being discarded. *B. discolor* fruit is juicy tasting. The pulp, with seeds removed, is used to make a refreshing drink, sometimes fermented to become alcoholic. *Z. mauritiana* fruit is a yellow berry, with a pleasant, thirst-quenching taste. The ripe fruits is crushed and soaked for some hours in water make a refreshing drink. *T. indica* has a brown and sticky fruit pulp which tastes like sherbet. Combining the pulp with sugar to taste and soaking it in cold water makes a refreshing drink, which is stirred well, and left to mature for a few days in a cool place (Tregold, 1986).

The study was carried out using the specific fruits, because of their local availability as wild fruits. The fruits used in the study also have a potential industrial use and it was therefore necessary to investigate the PG levels in case the fruits were ever to be used in industry.

2. Materials and methods

2.1. Samples

The fruits which were used in this study, *Z. mauritiana*, *U. kirkiana*, *T. indica* and *B. discolor*, were all in season during the time of the study and they were all purchased from a local market. The fruits were kept frozen (below 4 °C).

2.2. Extraction of polygalacturonase

The peels were first removed and the pulp separated from skin and seeds by pressing the seed and pulp mixture over cheesecloth. Pulp was then frozen.

The frozen pulp (200 g) was homogenized in a Waring blender with ice (200 g) until all the ice had melted. The pH of the homogenate was adjusted to 3 with 0.1 M HCI and the mixture centrifuged at 8000*g* in a Hermle centrifuge model Z640 at 4 °C for 20 min. The pellet was suspended in cold distilled water (180 ml), pH adjusted to 3 and the mixture centrifuged at 12,000 rpm in the Hermle centrifuge model Z640 at 4 °C for 5 min. Washing of the pellet by suspension in water at pH 3 was repeated until no reducing sugars were detected in the washings by the DNSA method (Chaplin, 1997; Minch, 1989).

After all the reducing sugars had been extracted from the pellet, cold extraction buffer (90 ml, l.7 M NaCl, 50 mM sodium citrate and 15 mM EDTA, pH 5.5) was added in order to release PG. The pulp was homogenized in a mortar and pestle in the presence of acid-washed sand. The mixture was incubated at 4 °C for 60 min with occasional stirring (Ali & Brady, 1992).

The solubilised enzyme was separated from the insoluble pulp by centrifugation at 1200 rpm, at 4 °C, in a Hermle centrifuge mode Z60 for 20 min. The supernatant, which was regarded as the crude enzyme extract, was tested for reducing sugars, using the DNSA method, and also for proteins, using the modified Lowry method. The solution of crude enzyme was dialysed against distilled water for 16 h with three changes of distilled water in order to remove excess NaCl and stored frozen at −20 °C until required.

2.3. Determination of protein in enzyme extracts

Protein was determined in all four enzyme extracts from the fruit by the Lowry and the BCA methods using bovine serum albumin (500 μg/ml) as the standard for calibration (Lowry, Rosebrough, Farr, & Randall, 1951; Peterson, 1983).

2.4. Measurement the activity of the polygalacturonase

The PG activity was determined by measuring the amount of reducing substances liberated from citrus pectin. The reaction mixture consisted of substrate buffer (0.125 g citrus pectin dissolved in 25 ml of 0.2 M acetate buffer, pH 4.2) and enzyme solution (0.5 ml). This reaction mixture was incubated at 37 °C for 30 min. Heating the mixture for 5 min in a boiling water bath then stopped the reaction. The amount of galacturonic acid was determined by measuring the reducing groups, using the 3,5, dinitrosalicylic acid method of Chaplin (1997). After cooling, absorbance was read at 570 nm in a UV Spectronic 20′ Genesys spectrophotometer. One unit of enzyme activity was defined as the activity that releases 1 mmol of reducing groups in 1 min at 37 °C (Sakai & Okushima, 1982).

For each enzyme preparation, the absorbance of three samples was measured against a galactose standard. From these absorbance values, the mean absorbance was calculated. This mean value was used to extrapolate the galactose concentration, which was used to calculate the enzyme activity.

2.5. The effect of pH on the activity of the polygalacturonase

The optimum pH of the PG was examined within the pH range 2–9. The different pH values were obtained by using the following buffers: 25 mM sodium acetate (pH

2.0, 3.0, 4, 4.5, 5.0), 25 mM phosphate Tris buffer (pH .0, 7.0) and 50 mM Tris buffer (pH 8.0, 9.0). For each enzyme preparation, the absorbance of five samples for each respective pH value was measured against a galactose standard. The galactose concentration was extrapolated, as above, as well as the calculation of the enzyme activity.

2.6. The effect of temperature on the activity of PG

The effect of temperature was determined by measuring the enzyme activity between 10 and 70 °C at the optimum pH for each enzyme from the four sources. The K_m and V_{max} of the PG were determined from the Lineweaver–Burk plot with citrus pectin as the substrate at optimum pHs and temperatures for the particular PG fruit extract.

2.7. Determination of the change in viscosity with enzyme activity

Viscosity reduction was estimated with 1% pectin, using a Viscometers UK viscometer in which viscosity was directly read off in centipoises at optimum pHs and temperatures of the enzymes from the four different sources.

2.8. Effect of metal ions on the activity of the polygalacturonase

The PG activity was determined at various concentrations of NaCl, $CaCl_2$, $MgCl_2$, and $BaCl_2$ from 0.1 to 1.5 mM, using commercial pectin as the substrate. The reaction mixture was incubated for 30 min at optimum pHs and temperatures of the enzymes from the four different sources. The reaction was stopped after 30 min by heating in a water bath. The DNSA method was used to determine the reducing sugars produced (Chaplin, 1997).

2.9. Isolation of pectic polysaccharide from Uapaca kirkiana

The ripe fruit was thawed and the peel and seeds removed. The pulp was macerated using a blender as far as possible and the suspension was centrifuged at 10,000*g* in a 13 HG Henmle ZK401 centrifuge as 40 °C for 20 min to obtain the juice extract. The resultant supernatant was collected and excess ethanol added to precipitate the polysaccharide. The precipitate was redissolved in the minimum amount of water and freeze-dried for 20 h. The polymer was kept in a desiccator at room temperature until required.

2.10. Investigation of the enzyme activity on Uapaca kirkiana polysacaccharide

Polysaccharide extract (0.025 g) was dissolved in water (50 ml) to make a substrate solution. The substrate solution (2 ml) was mixed with enzyme extracts from the four sources and incubated for 40 min at 37 °C. The reaction was stopped by heating in a boiling water bath for 5 min. Reducing sugars were determined using the DNSA method. This experiment was done to see whether the PG from different sources could hydrolyse pectic material from different sources.

2.11. Statistical analysis

Statistical analysis (ANOVA and *t*-test) of data was carried out using the statistical software Graghpad INSTAT.

3. Results and discussion

The PG was extracted from all four fruits used in the project. The presence of the pectin-degrading enzymes is routinely estimated on the basis of specific activity. The amount of protein is related to the enzyme activity. In the crude enzyme extracts, the presence of protein is a good indication of the presence of enzyme. Ripe *U. kirkinia* fruit which recorded the highest enzyme activity also, had the highest protein concentration, as shown in Table 1. The colouration in the Lowry method used for protein determination is, however, prone to interference by pectin. The pectin interacts with the reagents used in the Lowry produced by promoting the development of a significant colouration, linearly related to the amount of pectin, affecting the absorbance.

As shown in Fig. 3, viscosity of 1% pectin solution is reduced with time, indicating the hydrolysis of pectin by the enzyme from all the sources. The Lineweaver–Burk plot in Fig. 4 was used to calculate K_m and V_{max} of the PGs from the four different sources, which are shown in Table 3. The K_m values of PG varied from 0.12 to 0.25 mg/ml, values that were comparable to the K_m of fungal PG, which was from 0.09 to 3.2 mg/ml (Kester, Kusters-Van Someren, Muller, & Visser, 1996; Nozaki, Miyairi, Hozumi, Fukui, & Okuno, 1997).

As shown in Table 2, *Z. mauritiana* fruit also had a high PG activity (after ripe *U. kirkiana* fruit). *Z. mauritiana* is also soft and there is a direct correlation be-

Table 1
Protein concentrations for the different crude enzyme preparations extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruits

Sample	Protein concentration (mg/ml)
U. kirkiana (unripe)	0.62 ± 0.21
U. kirkiana (ripe)	1.97 ± 0.13
Z. mauritiana	1.21 ± 0.24
B. discolor	0.82 ± 0.17
T. indica	1.11 ± 0.28

Results are means of at least three determinations.

Table 2
Enzyme activity of the crude enzyme extracts extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruits

Sample	Enzyme activity ($mmol\,min^{-1}\,mg^{-1}$)
U. kirkiana (unripe)	1.99 ± 0.13
U. kirkiana (ripe)	6.64 ± 0.38
B. discolor	3.23 ± 0.12
Z. mauritiana	5.45 ± 0.19
T. indica	4.56 ± 0.27

Results are means of at least three determinations.

Table 3
V_{max} and K_m values for the polygalacturonase from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor*

Sample	V_{max}	K_m
U. kirkiana (unripe)	0.0091	0.123
U. kirkiana (ripe)	0.0119	0.152
B. discolor	0.0057	0.115
Z. mauritiana	0.0106	0.171
T. indica	0.0105	0.133

tween the PG, activity and fruit softening. However, the fruit of *Z. mauritiana* is not as soft and fleshy as the ripe *U. kirkiana* fruit and this probably explains the fact that PG is lower in *Z. mauritiana* than in ripe *U. kirkiana* fruit.

T. indica fruit followed *Z. mauritiana* in PG activity. The presence of the PG is an indication of the soft nature of the pulp and thus the PG played a role in this softening. The extent of this softening is not, however, intense and this probably explains the lower PG activity in the fruit. The *T. indica* pulp is sticky in appearance, which is characteristic which is characteristic of pectin polymers or pectic substances. These are degraded by PG and this explains the presence of the PG activity in the *T. indica* fruit.

The *B. discolor* fruit had the lowest PG activity among the ripe fruits analysed. The fruit in its ripe state is considered dry. Dry fruits and other dry parts of the fruits do not contain pectin but a precursor called pectose. the PG is not linked to this precursor and this probably explains the low the PG activity in the *B. discolor* fruit. As the activities of the PG decrease, the fruit dries. Fruit drying is linked to a phase in pectin formation after the fruit has completely matured. At this stage, pectin substances are in the form of protopectin and this is not as soft and fleshy. All the other parts of the fruit are hard and dry due to the low activity of the PG recorded.

A major structural change is the degradation of polyuronides by the increased PG activity and a concomitant loss of galacturonic acid residues from the cell wall (McReady & McComb, 1985). When the fruit becomes mature, it yields moreor less pectin until the time when the parts undergo their final breakdown (Crookes & Grierson, 1983). The pectin decomposes to sugars of simpler constitution or to other substances no longer possessing the water absorbent and swelling properties of pectin.

The increased pectin decomposition of fruits as they progress from the green state to the ripe state explains the increased activity of the PG in ripe *U. kirkiana* fruit. The increased activity leads to the fleshy and soft appearance of ripe fruits. On the other hand, unripe fruits do not contain pectin but a precursor of pectin, which yields pectin on boiling with dilute acids. This precursor is referred to as pectose. It cannot be degraded by the PG (Crookes & Grierson, 1983). Low activity in unripe *U. kirkiana* fruit can also be explained in terms of the first phase of pectin formation in fruits and plants in general (Ben-Arie et al., 1979).

The study showed that the optimum pH was different for each fruit, varying from 4.0 and 5.5, as shown in Fig. 2. Polygalacturonase, in general, has been found to have an optimum pH of between 4.0 and 4.5 (Tucker & Grieson, 1997). The results, however, show that the optimum pH varies from source to source and also from fruit to fruit. The optimum pH, however, does not indicate the pH at which the enzyme is most stable. It only represents the pH at which the enzyme works best.

As shown in Fig. 1, the optimum temperature of the isolated enzyme ranged from 25 to 37 °C. A similar trend for optimum pH and optimum temperature was observed by Isshiki, Akimitsu, Ishii, and Yamamoto (2000) and Ali and Brady (1992), for the PG from pear scab pathogen, *Venturia nashicola* and *Venturia pirina* and the PG from tomatoes.

Figs. 5–8 illustrate the effects of varying concentrations of metal ions on the activity of the PG from the four sources. The PG was activated more by the

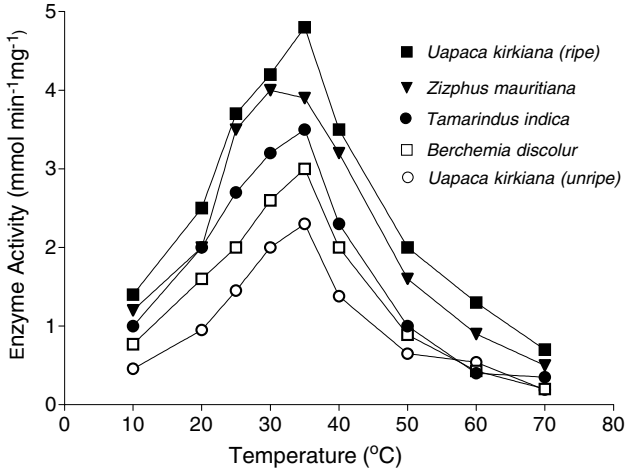

Fig. 1. Effect of temperature on enzyme activity of the polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit. The activity of the crude polygalacturonase on 1% pectin solution was measured at different temperatures from 10 to 70 °C.

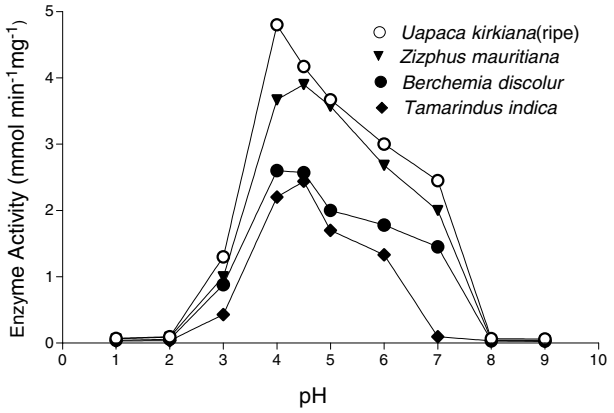

Fig. 2. Effect of pH on enzyme activity of the polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit. The pH of the solution of the crude enzyme and 1% pectin solution was adjusted to the pHs indicated with a buffer and incubated at the different optimum temperatures for the polygalacturonase from the four different sources for 30 min. The reducing groups were determined by the DNSA method.

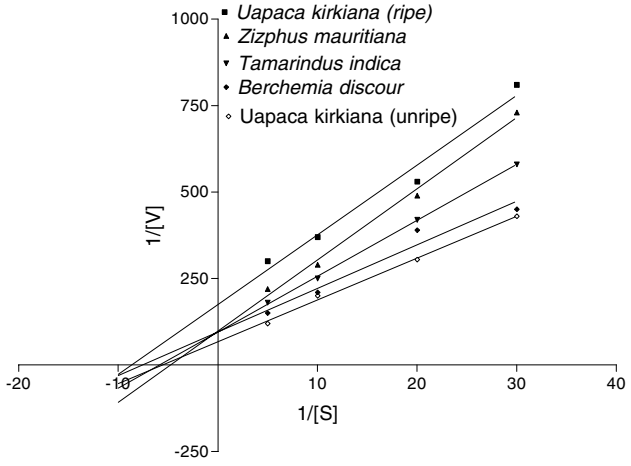

Fig. 4. Lineweaver–Burk plot of substrate (pectin) concentration vs intial velocity. The polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit was incubated at the optimum temperatures and pHs for the different enzyme extracts. The velocity (V) was the increase of reducing groups in mmol/min/ml protein. The substrate concentration is in mg/ml. The reducing groups were determined by the DNSA method.

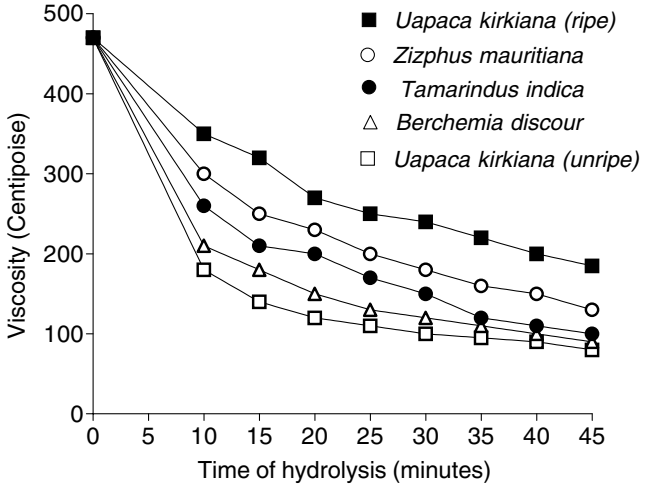

Fig. 3. Change in viscosity of a 1% pectin solution with degradation by crude polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruits. Vicosity was at graded times.

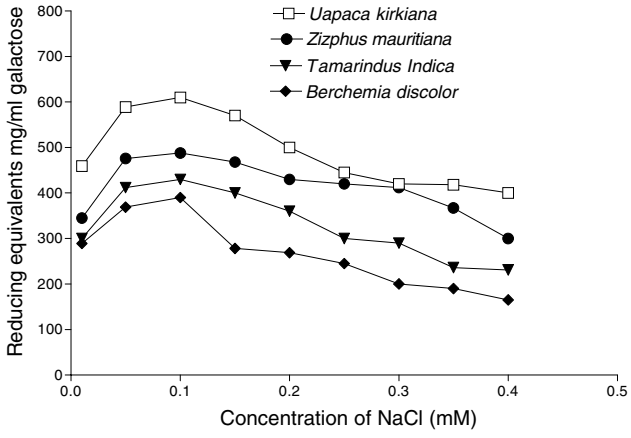

Fig. 5. Effect of concentration of NaCl on the activity of the polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit. The polygalacturonase activity was determined at various concentrations of NaCl, from 0.1 to 1.5 mM using commercial pectin as the substrate. The reaction mixture was incubated for 30 min at optimum pHs and temperatures of the enzymes from the four different sources. The reducing groups were determined by the DNSA method.

monovalent ions (Na+). The ions are believed to activate the enzyme substrate complex. These ions presumably bind to the enzyme, inducing conformational orientation favouring substrate binding. Further increases in Na+ ions become inhibitory to the substrate binding.

From Fig. 6 it can be seen that a concentration of $CaCl_2$ below 0.01 mM stimulates the PG. As the concentration of the divalent cations is further increased, the enzyme activity is decreased. The cause of the inhibition is that calcium ions enhance the gelling of the pectin molecules, thereby producing interlinking and making the substrate inaccessible to the enzyme.

The PG activity was unaffected by magnesium ions up to 30 mM but was inhibited by 40 mM $MgCl_2$, as shown Fig. 7 while $BaCl_2$ was inhibitory at concentrations of 1 mM and higher, as shown in Fig. 8. The inhibitory effect of the divalent cations was probably a result of physical interactions with the pectin and not a direct effect on the enzyme (Walter, 1991).

Polygalacturonase has many applications in the food industry. However, the PG from the other three fruits showed very little activity on the polysaccharide from

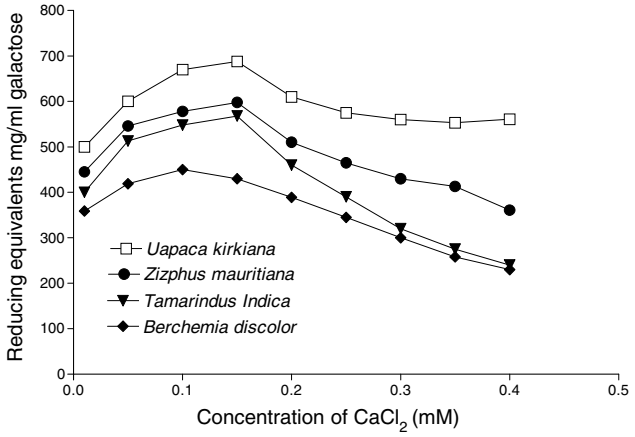

Fig. 6. Effect of concentration of $CaCl_2$ on the activity of the polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit. The polygalacturonase activity was determined at various concentrations of $CaCl_2$ from 0.1 to 1.5 mM, using commercial pectin as the substrate. The reaction mixture was incubated for 30 min at optimum pHs and temperatures of the enzymes from the four different sources. The reducing groups were determined by the DNSA method.

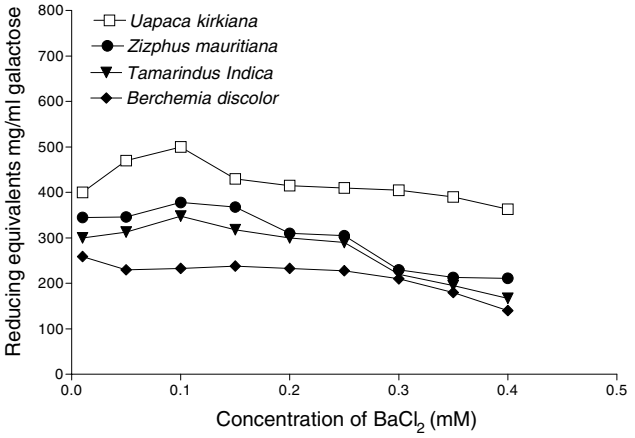

Fig. 8. Effect of concentration of $BaCl_2$ on the activity of the polygalacturonase, extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit. The polygalacturonase activity was determined at various concentrations of $BaCl_2$, from 0.1 to 1.5 mM, using commercial pectin as the substrate. The reaction mixture was incubated for 30 min at optimum pHs and temperatures of the enzymes from the four different sources. The reducing groups were determined by the DNSA method.

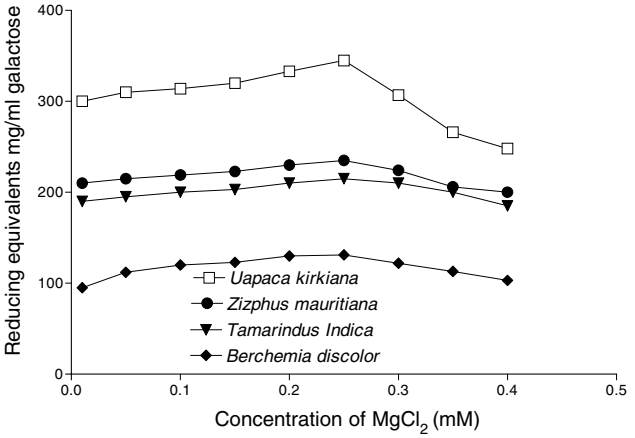

Fig. 7. Effect of concentration of $MgCl_2$ on the activity of the polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit. The polygalacturonase activity was determined at various concentrations of $MgCl_2$, from 0.1 to 1.5 mM, using commercial pectin as the substrate. The reaction mixture was incubated for 30 min at optimum pHs and temperatures of the enzymes from the four different sources. The reducing groups were determined by the DNSA method.

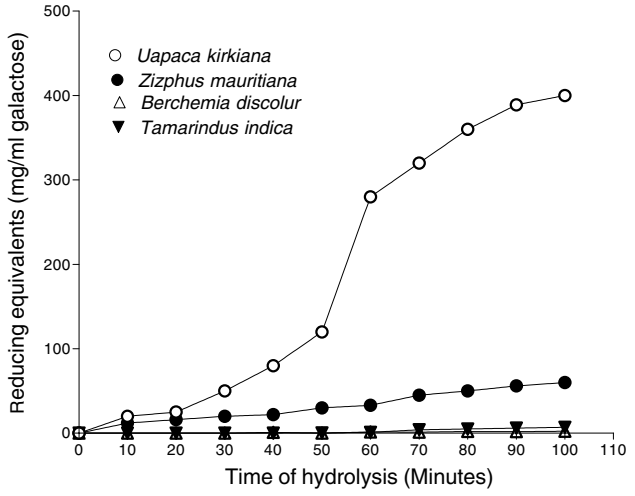

Fig. 9. Investigation of the action of polygalacturonase extracted from *U. kirkiana*, *Z. mauritiana*, *T. indica* and *B. discolor* fruit on a pectic polysacharide extracted from *U. kirkiana.* This experiment was done to see whether the PG from different sources could hydrolyse pectic material from different sources. Polysaccharide extract was dissolved in water to make a substrate solution. The substrate solution was mixed with enzyme extracts from the four sources and incubated for 40 min at 37 °C. The reducing groups were determined by the DNSA method.

U. kirkiana fruit, as shown in Fig. 9, suggesting that there may be different isoforms of PGs from the four different sources.

4. Conclusion

All the fruits analysed in the study showed significant PG activity of PG. Ripe *U. kirkiana* fruit, which had the highest the activity, is a potential source of PG for industrial applications. Studies to purify the PG extracted from local fruits, particularly the *U. kirkiana* fruit, will help characterize the PG further if it is to be used in the industry. It is also worthwhile to establish whether the molecular weight of the PG varies from source to source and in this case from fruit to fruit.

Acknowledgements

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