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EXO-POLYGALACTURONASE OF APPLE

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Abstract—Exo-polygalacturonase was extracted from apple cortical tissue. The enzyme hydrolyses polygalacturonic acid and has a pH optimum of 4.5–5 with this substrate. It is inhibited by EDTA and citrate and is activated by Ca^{2+} and to a lesser extent by Sr^{2+}. The enzyme which has a MW of 58 000 degrades apple cortical cell wall preparations releasing low MW uronic acid residues and polyuronide.

INTRODUCTION

Softening of the cortical tissue of ripening apples is characterised by the loss of galactose residues from the cell wall and an increase in the polygalacturonide which is freely soluble in aqueous solutions [1, 2]. It is thought that the loss of galactose residues arises from hydrolysis of the galactan of the primary cell wall by β-galactosidase (EC 3.2.1.23) and that the soluble polyuronide derives from the middle lamella region of the wall [3–5].

Endo-polygalacturonase (EC 3.2.1.15) and exo-polygalacturonase (EC 3.2.1.67) are both present in ripening peaches [6] and pears [7]. It has been suggested that the endo-enzyme catalyses the solubilisation of polyuronide and the softening of the tissue observed in these fruits during ripening but no role has been assigned to the exo-polygalacturonase [7]. Endo-polygalacturonase is not present in ripening apples [8] and therefore the mechansim of solubilisation of polyuronide in this fruit is different from that suggested for peaches and pears. This paper describes the identification and partial purification of the exo-polygalacturonase (exo-PG) of apple cortical tissue and some of its properties.

RESULTS AND DISCUSSION

Identification of polygalacturonase

The polygalacturonase of apple was classified as a terminal cleaving (exo) enzyme from (a) viscometric data and (b) TLC of the reaction products [8]. (a) Apple PG (0.36 U/g fr. wt of tissue) reduced the viscosity of polygalacturonic acid (PGA) by only 3% in a 5 hr incubation. In contrast a polygalacturonase preparation from pear (0.58 U/g fr. wt) which contains an endo-PG (random cleaving enzyme) in addition to the exo-enzyme [7] reduced the viscosity of PGA by 40% in 4.5 hr. (b) The reaction products were separated using TLC. Galacturonic acid was the only reducing substance detected in samples taken after 5 and 23 hr. It was not detected in 0 hr samples.

Table 1. Partial purification of exo-PG

	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein	Yield (%)
Initial preparation	60.0	3 97	10 40	0 38	100
Polyethylene glycol	18 1	3 89			98
$(NH_4)_2SO_4$, 0–94%*	4 0	3 28			83
Sephadex G-100 pooled peak†	11 5	1 26	0 26	4 85	32
Polyethylene glycol	5 7	1 31			33

* 17 ml of preparation treated with $(NH_4)_2SO_4$. †3 ml of PG preparation applied to column.

Properties of PG

The maximum rate of reaction was observed at a concentration of 0.5 mg PGA/ml. The K_m value for the enzyme with this substrate was 0.012 mg/ml [9]. The optimum pH for the hydrolysis of PGA by PG was 4.5–5. The enzyme displayed half maximal activity at pH 4.2 and 5.6.

Apple exo-PG was activated 32% by 0.1 mM Ca^{2+}. Sr^{2+} (0.1 mM) activated the PG by only 12.5% whilst Mg^{2+} and Ba^{2+} were slightly inhibitory at this concentration. EDTA and citrate at final concentrations of 5 mM inhibited the exo-PG by 56 and 30.5% respectively indicating that a divalent cation may be required for enzyme activity [6].

Partial purification of PG

Apples have a characteristically low protein content, *ca* 0.2% by wt [10] and therefore enzymes prepared from apple tissues have to be concentrated before their purification can be attempted. A number of methods were tested including ultrafiltration, concentration in dialysis tubing with sucrose and also Sephadex G-25 and G-200 beads [11] and dialysis against a solution of polyethylene glycol. The last method gave a good recovery of PG activity (Table 1) and raised the enzyme concentration sufficiently to enable the use of $(NH_4)_2SO_4$ in a second concentration step. The concentrated PG preparation was fractionated by gel filtration using Sephadex G-100 and PG eluted as a single peak with a recovery of 70% of the applied activity. Fractions 32–36 were pooled and concentrated.

Substrate specificity

Exo-PG released only 0.07 μmol of reducing groups per ml of incubation from polymethylgalacturonic acid (53% esterification of carboxyl groups) in 3 hr whilst the enzyme released 0.28 μmol of reducing groups per ml from PGA, at the same concentration, in the same period. The results indicate that the enzyme preferentially hydrolyses α-1,4-linkages between non-esterified galacturonsyl residues. The hydrolysis of the galacturonan by exo-PG will also be influenced by the 2-rhamnosyl residues in the polymer and the presence of araban and galactan covalently linked to the galacturonan [12, 13].

Molecular weight

The MW of PG was estimated by gel filtration to be 58 000.

Degradation of cell walls by exo-PG

The ability of the exo-PG to release uronic acid residues from cell wall preparations in *in vitro* incubations (Tables 2, 4) suggests that the enzyme is responsible for the loss of galacturonic acid residues from the cell walls of stored apples (Table 3). Pectinesterase (EC 3.1.1.11) was associated with the cell wall preparations (1.2–2 U/g fr. wt of tissue) and if this wall-bound enzyme is active at pH 4.5 then it could stimulate the release of uronic acid residues from the cell walls catalysed the the exo-PG.

Table 2. Solubilization of uronic acid residues from apple cortical cell wall preparation 2 by exo-PG

Time (hr)	Uronic acid residues released (μg anhydro sugar/ml) Cell walls + PG	Cell walls − PG
0	2.08	0.24
22	87.76	6.08
51.5	140.80	7.28
72	142.40	8.64

The composition of cell wall preparation 2 is given in Table 3. There were no uronic acid residues in the enzyme preparation.

Table 3. Composition of cortical cell walls, prepared from apples ripened in 2% oxygen [2], used in the degradation studies with exo-PG

Cell wall preparation	Time in store (days)	Composition of cell wall Galactose	Uronic acid (mg anhydro sugar/g fr wt)	Soluble polyuronide
1	0	2 75	3 48	0 10
2	79	1 26	2 93	0 13
3	119	0 97	2 75	0 35

No changes were observed in other neutral sugar components of the cell wall during ripening of the fruit [2].

Table 4. Degradation of apple cortical cell wall preparations 1–3 by exo-PG

Cell wall preparation	Uronic acid residues released (mg anhydro sugar g fr. wt in 72 hr	Uronic acid released as % of uronide content of cell wall
1	0.57	16.4
2	0 51	17.4
3	0.43	15.6

Table 5. Composition of reaction products released by PG during incubation with cell wall preparations 1–3*

Component	Anhydro-sugar content (mg g fr wt) Polyuronide (insoluble in EtOH) 1	2	3	Products soluble in EtOH 1	2	3
Rha	0 04	0 05	0 04	0 04	0 03	0 02
Ara	0 03	0 02	0 02	0 06	0 09	0 08
Xyl	0.08	0 08	0 05	0 07	0 06	0 06
Gal	0	0	0	0 52	0 12	0 08
Uronic acid	0 14	0 13	0 09	0 43	0 38	0 34

* EtOH (3 vol.) was added to the soluble reaction products and the composition of both the alcohol soluble material and the polyuronide (insoluble in alcohol) determined

When ethanol [6] was added to the soluble products released by exo-PG from cell wall prepatations 1, 2 and 3, the precipitate, polyuronide, contained 23.1, 25.7 and 20.3% respectively of the uronic acid residues solubilized from these wall preparations by the enzyme. The ethanol soluble material was fractionated by gel filtration using Sephadex G-25. The uronic acid residues were eluted from the column as a single peak and in the same volume as galacturonic acid. The results in Table 5 show that *ca* 19% of the galactose residues of cell wall preparation 1 were released during the incubation with exo-PG compared with *ca* 9% from the other two wall preparations which have much lower galactose contents (Table 3). Xylose (*ca* 20% of the total) and arabinose residues (*ca* 6%) were also released [2].

Arabinose and xylose residues were associated with the polyuronide (Table 5). Whilst the arabinose is probably covalently linked to the polyuronide [13], xylose is not a component of the galacturonans [13] and it is thought that these residues constitute a xylan which is co-precipitated with the polyuronide on the addition of alcohol. Ethanol precipitates hemicelluloses as well as PGA and polymethylgalacturonic acid [14].

The galactose residues released are thought to arise from hydrolysis of the galactan of the cell wall by β-galactosidase [1] which was present in the wall preparations and in the exo-PG preparation (unpublished data). β,D-Xylosidase (EC 3.2.1.37) and α,L-arabinofuranosidase (EC 3.2.1.55) assayed using the nitrophenyl derivatives of xylose and arabinose, respectively, were also present in the partially purified PG preparation (unpublished data) and these glycosidases could be responsible for the observed release of xylose and arabinose residues in the incubations.

Polyuronide is solublized as apples soften during ripening. The highly esterified polymer is thought to derive from the middle lamella region of the cell wall [3, 4]. Assuming that the exo-PG is localized in this area of the wall then solubilization of the highly esterified polyuronide molecules could arise from a loosening of the polyuronide network following hydrolysis by exo-PG of galacturonans with no or only a low degree of esterification.

EXPERIMENTAL

A bulk sample of apples was obtained on 15 September 1975 from trees of *Malus domestica* Borkh. cv Cox's Orange Pippin grown at East Malling Research Station and stored in air at 3.3°. The apples were used after 5–8 months in store. Cox's Orange Pippin apples grown in New Zealand in the 1975/75 season and shipped to the UK in April 1976 were stored as above. The fruit was used after 2–6 months in store. Conference pears were harvested on 11 September 1975 and stored in air at 0°. They were transferred to 20° for up to 8 days before use. The preparation of the cell walls used here is described in an earlier paper [2].

Preparation of enzyme. Cortical tissue of apple was homogenized in Me_2CO, 0.2 M Tris–HCl, pH 8.9 (9:1) at 2° [1]. The residue, collected by filtration, was resuspended in 5 mM Na–Pi (pH 7.2) at 2 ml/g tissue and kept under toluene at 2° for 3 days. The suspension was filtered and the residue resuspended in 5 mM Na–Pi, 0.75 M NaCl (pH 7.2). After standing for 15 min at 2°, with occasional stirring the suspension was filtered and the filtrate dialysed against 5 mM Na–Pi (pH 7.2) for 150 min. The dialysate was centrifuged at 27 000 *g* for 10 min at 5°. The supernatant constituted the initial PG prep. PG was stable for at least 5 days at 2°. A 4-fold increase in PG extracted by the high ionic strength buffer was achieved by increasing the period of suspension of Me_2CO insoluble material in 5 mM Na–Pi (pH 7.2) from 1 hr to 3 days. PG was prepared from the cortical tissue of pears using the above procedure.

Enzyme purification. The initial PG prep. (60 ml) was concd by dialysis against 10% (w/v) polyethylene glycol, MW 20000 (Aquacide 3, Calbiochem Ltd.) in 5 mM Na–Pi, 0.1 M NaCl (pH 7) for 16 hr at 2°. Solid $(NH_4)_2SO_4$ was added slowly to the dialysate with constant stirring at 20° to 94% satn. After standing a further 60 min at 2°, the soln was centrifuged at 27000 *g* for 10 min at 3°. The pellet was dissolved in 4 ml of 5 mM Na–Pi, 0.1 M NaCl (pH 7) and the soln dialysed for 18 hr against the same buffer at 2°. The dialysate was centrifuged at 27000 *g* for 10 min at 3°, and 3 ml of the supernatant were applied to a column (39 × 2.5 cm) of Sephadex G-100 which had previously been equilibrated with 5 mM Na–Pi, 0.1 M NaCl (pH 7). Flow rate of the column was 10.8 ml/hr and 2.7 ml fractions were collected. The exo-PG was eluted as a single peak and fractions 32–36 pooled. The pooled peak was concd by dialysis against polyethylene glycol in buffer, as before. For the viscometric assays, the initial prepn was concd *ca.* 2.7 fold (U/g fr. wt) by ultrafiltration using a PM-10 membrane (Amicon Corp.). PG activity prepared from pears was concd *ca* 2 fold (U/g fr. wt), prior to assay, in dialysis tubing with sucrose. Dialysis against 5 mM Na–Pi, 0.15 M NaCl (pH 7) was used subsequently to reduce the sucrose concn of the prepn.

Substrates. Citrus PGA and polymethylgalacturonic acid were refluxed with 80% EtOH, filtered and dried in air prior to use. The uronic acid contents of the polysaccharides were 81 and 77% by wt respectively and the degree of esterification of polymethylgalacturonic acid was 53% (M. Knee, personal communication).

Assay of PG. The reaction mixture consisted of 1 ml of PG prepn and 1 ml of PGA (1 mg/ml) in 0.2 M NaOAc (pH 4.5) and the incubations were at 25°. Reducing groups released were measured by the method of ref. [15] as modified in ref. [16]. Controls in which either the substrate or the enzyme prepn were omitted were also included. The release of reducing groups was linear with time during the period of the assay and directly proportional to the concn of enzyme present. A unit of enzyme activity (U) is defined as the amount of enzyme which released 1 μmol of reducing groups per hr. Transeliminative cleavage of the glycosidic linkages between esterified uronic acid residues is catalysed by alkali and generates reducing groups, particularly at high temp. [8]. Therefore, when polymethylgalacturonic acid was used as substrate for PG, samples from the incubations were mixed with alkaline Cu reagent [16] and allowed to stand for 2 hr at 20° to ensure de-esterification of the substrate before proceeding with the heating step. Activation of PG by divalent cations was tested using a PG prepn concd *ca* 3 fold (U/g fr. wt) by dialysis against polyethylene glycol. The reaction mixture consisted of 0.5 ml of PGA (1 mg/ml) in buffer, 0.1 ml divalent cation soln and 0.4 ml of enzyme prepn. In the later stages of the work, Ca^{2+} were included in the assay of PG activity at a final concn of 0.1 mM. PG was assayed viscometrically using PGA (10 mg/ml) in 0.2 M NaOAc. The pH of the substrate soln was raised to pH 4.5 with M-NaOH and the soln clarified by centrifugation at 1000 *g* for 5 min. The reaction mixture consisted of 0.5 ml of PG prepn and 2.5 ml of PGA. The change in viscosity of the incubation was measured with an Ostwald viscometer at 25°. Controls in which PG was omitted showed no change in viscosity The reducing groups released were assayed in separate incubations, as above, because of the small vol. of prepn available. The substrate concn (0.5 mg/ml) was sufficient to saturate the pear PGs and the apple PG.

Assay of pectinesterase in cell wall preparations. Polymethylgalacturonate of the wall prepns was used as the substrate for the assay [17]. Pectinesterase was measured by determining the amount of 0.02 M NaOH required to maintain pH 7.5 of a mixture of 10 ml of cell wall prepn plus 10 ml of H_2O and a final concn of 0.1 M NaCl. The incubation was at 20°. A Unit of pectinesterase activity (U) is defined as 1 μequivalent of ester hydrolysed per min.

Buffers. Acetate (0.2 M) and Pi (0.1 M) buffers, pH 4–7, were used in the determination of pH optimum of PG. PGA was not soluble below pH 4.

MW determination. The MW of the PG was determined by gel filtration on Sephadex G-100. The value was calculated from the elution vols of cytochrome *c*, chymotrypsinogen, ovalbumin and BSA [18].

Protein estimation. Protein content was estimated using the fluorimetric procedure of ref. [19]

TLC of reaction products. Incubation of PG with PGA was terminated by heating at 95° for 5 min. EtOH (95%, 2 vol.) was added and the undegraded PGA removed by centrifugation at 1000 *g* for 5 min. The vol. of the supernatant soln was reduced at 35° to 0.5 ml. The soln was chromatographed on cellulose [20] and reducing substances detected using alkaline $AgNO_3$ [21].

Incubation of PG with cell walls. The cell walls were prepared from apples ripening in 2% O_2 at 3.3° after different periods in store [2] and held in H_2O at −20° until required. The cell wall prepns were thawed, washed with H_2O and resuspended in 0.2 M NaOAc (pH 4.5) to 2 ml/g. Starch was not removed from the cell walls prior to incubation with PG. The reaction mixture consisted of 2.5 ml of cell wall prepn in buffer, 0.5 ml of $CaCl_2$ (1 mM) and 2 ml of PG prepn (0.62–0.76 U). The mixture was incubated at 20°, under toluene, with continuous agitation. (Controls in which cell walls or enzyme were omitted were also included). The release of uronic acid residues was followed with time. Aliquots of the incubation were diluted with 3 vol. of 67 mM NaOH and left to stand for 30 min at 20°, to de-esterify soluble polymers. The uronic acid residues present were assayed using the carbazole H_2SO_4 reagent [22]. The incubation was terminated by filtration through glass fibre paper and the filtrate retained. EtOH (3 vol.) was added to the filtrate and the soln allowed to stand 18 hr at 2°, prior to centrifugation at 1000 *g* for 20 min. The pellet was dissolved in 50 mM NaOH. The uronic acid contents of the supernatant and the dissolved pellet were assayed colorimetrically. The vol. of the EtOH soluble material (4.5 ml) was reduced *in vacuo* at 35° to *ca* 0.1 ml and 1.5 ml of 0.1 M NaOAc (pH 4.5) added. This soln was fractionated by gel filtration on a Sephadex G-25 column (8 × 1.5 cm), at 2°, equilibrated with the NaOAc buffer. Column flow rate was 10.8 ml/hr and 0.9 ml fractions were collected. The uronic acid content of the fractions were assayed colorimetrically.

Hydrolysis of reaction products. The reaction products from incubations of cell walls with PG were fractionated using EtOH, as above. The EtOH insoluble material was washed with Et_2O and allowed to dry. H_2SO_4 was added to 0.4 M. After hydrolysis at 121° for 60 min the soln was neutralised with $BaCO_3$, evapd to 40° and dried over P_2O_5 *in vacuo*. The EtOH soluble material was reduced in vol. *in vacuo* at 35° to *ca* 0.1 ml and 1 ml H_2O

added. The soln was passed through a column (2 × 1 cm) of Dowex 50 – ×8 (H^+ form) resin, concd and H_2SO_4 added to 0 4 M. Hydrolysis was performed as above.

GLC of monosaccharides. Neutral sugars in the hydrolysates were analysed as their alditol acetates [3]. The derivatives were injected onto a column (1.8 m × 4 mm id) of 5% OV-275 on Chromosorb W using an injection temp. of 275°, column temp of 225° and N_2 carrier at 40 ml/min

Uronic acid content of cell walls The uronic acid content of the wall prepns was determined colorimetrically following incubation with pectinase [23] The values were corrected for the presence of glucose, arising from hydrolysis of the starch in the prepns, by also assaying the products with sulphonated α-naphthol reagent [22] and solving simultaneous equations derived from the contributions of uronic acids and neutral sugars (assumed to be glucose) to the *A* measured in the two assays (M. Knee, personal communication).

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