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Use of crosslinked mucilage prepared from ruredzo (*Dicerocaryum zanguebarium*) in the purification of polygalacturonase extracted from tomato

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The mucilage from ruredzo (*Dicerocaryum zanguebarium*) was crosslinked in alkaline conditions with epichlorohydrin. Polygalacturonase (PG) was extracted from ripe tomatoes by first macerating at pH 3 at low ionic strength. After washing off sugars, the enzyme was extracted in buffer containing 1.7 M NaCl. PG was bound to crosslinked mucilage (CLM) at pH 4 and released by eluting with buffer containing 1 M NaCl after washing off unbound proteins. A six-fold purification of PG was achieved. The purified enzyme showed two main bands of molecular weights 30 000 and 44 000. Used CLM was regenerated by treatment in 1 M NaCl and 1 M HCl. Treatment of PG with regenerated CLM produced results that were similar to the purification with fresh CLM. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

There is an increased trend towards the use of renewable resources including plant materials. We have been trying to find applications for the mucilage that is isolated from ruredzo. If suitable uses can be identified, we believe that a plentiful supply of the material would be available even when relying on the naturally growing plant as the only source of the mucilage.

Ruredzo is a plant that grows widely in the sandy soils of many parts of Southern Africa. The creeping plant has traditionally been used for a variety of purposes that depend on the presence of a slimy material in the leaves (Benhura & Marume, 1993). The mucilage from ruredzo is a pectic material with a molecular weight of about 500 000 and has been shown to contain 8% uronic acid groups.

Crosslinked pectins have been used in the purification of polygalacturonase (PG), a cell wall enzyme that is responsible for the softening of vegetable material such as tomatoes (Inoue *et al.*, 1984). The purification methods are based on the ability of crosslinked pectate to act as an affinity adsorbent for PG. After the enzyme is bound to the matrix, environmental conditions such as pH and ionic strength can be changed in order to elute the enzyme (Kohn *et al.*, 1976).

At present, the purification of pectic enzymes involves several steps in order to produce a preparation of acceptable activity. One attraction of affinity chromatography is that substantial purification of proteins may be effected in a single step. Reduction in the number of steps that are involved in the purification of a given enzyme would be expected to reduce the cost of preparation of that enzyme.

The purpose of this study was to investigate the use of crosslinked ruredzo mucilage (CSM) as an affinity adsorbent in the purification of PG from tomatoes. The procedure would not only provide an application for ruredzo mucilage but it would also effect purification of the enzyme in a single step.

MATERIALS AND METHODS

Extraction of PG

Soft ripe tomatoes were purchased from a local supermarket and stored frozen at −20°C. Tomatoes, chilled and sliced (200 g) were homogenized in a Waring Blender in 200 g of ice until all the ice had melted. The pH of the homogenate was adjusted to 3 with HCl and the mixture centrifuged at 8000 rpm in an ICE centrifuge for 20 min. The pellet was suspended in 180 ml of cold

distilled water with pH adjusted to 3 and the mixture centrifuged at 12000 rpm in the ICE centrifuge for 5 min (Minch, 1989). Washing of the pellet by suspension in water at pH 3 was repeated until no reducing sugars were detected in the supernatant using the 3,5-dinitrosalicylic acid (DNSA) method (Chaplin, 1986).

After all the reducing sugars had been extracted from the pellet, 90 ml of cold extraction buffer (1.7 M NaCl, 50 mM sodium citrate, 15 mM EDTA, pH 5.5) were 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 for 60 min at 40°C with occasional stirring. The solubilized enzyme was separated from the insoluble pulp by centrifugation at 12000 rpm at 4°C for 20 min (Ali & Brady, 1982). The supernatant, which was regarded as the crude enzyme extract, was tested for reducing sugars and protein content. The solution of crude enzyme was dialysed for 16 h with three changes of distilled water in order to remove excess NaCl from the extraction buffer. The solution of crude enzyme was stored frozen at −20°C.

Determination of reducing sugars

To samples, standards and controls (100 μl), 1 ml of the DNSA reagent was added and the mixture heated in a boiling water bath for 10 min. The mixtures were cooled rapidly to room temperature under running tap water. The absorbance was measured at 570 nm (Chaplin, 1986).

Protein determination

Protein was measured following the modified Lowry method with the enzyme preparation being diluted where necessary; bovine serum albumin was used as the standard for calibration (Peterson, 1983).

Measurement of PG activity

PG activity was determined by measuring the amount of reducing substance 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 ml) and solution of enzyme (0.5 ml). After incubation at 37°C for 30 min, the reaction was stopped by heating the mixture for 5 min in a boiling water bath. The amount of galacturonic acid was determined by measuring the reducing groups using the DNSA method. One unit of enzyme activity was defined as the activity which liberated reducing substance corresponding to 1 μmol D-galacturonic acid per millilitre of reaction mixture at 37°C in 30 min (Sakai & Okushima, 1982).

The effect of pH on the activity of PG

PG activity was determined at pH values between 3 and 7. Citrus pectin (0.125 g) was dissolved in 25 ml of either 0.2 M acetate buffer or 0.1 M citrate–0.2 M phosphate buffer at pHs between 3 and 7 and left to hydrate overnight in the refrigerator at 4°C to give the substrate buffer. The solution of crude enzyme was thawed and adjusted to the appropriate pH using the 2 M buffer constituents. The enzyme activity was determined as described above.

Preparation of crosslinked ruredzo mucilage

Crosslinking of mucilage with epichlorohydrin (ECH) was done in both homogeneous and heterogeneous modes. In the heterogeneous mode, purified dried mucilage was treated with ECH; in the homogeneous mode, a solution of crude mucilage was used in the crosslinking procedure (Kunaik *et al.*, 1972). The mucilage was isolated and purified as described previously (Benhura & Marume, 1993).

Heterogeneous crosslinking of mucilage

In the routine procedure for crosslinking using the heterogeneous mode, mucilage (10 g), 40% aqueous dimethyl sulphoxide (DMSO, 150 ml), epichlorohydrin (ECH, 40 ml), and 5 M NaOH (50 ml) were mixed in a stoppered 250 ml Erlenmeyer flask (Hatanaka *et al.*, 1990). The reaction mixture was incubated at 40°C in a shaking incubator (Innova 4300; New Brunswick Scientific) at 200 rpm, for 2 h. The precipitated CLM was washed with distilled water until neutral. The CLM was suspended in 50 volumes of hot distilled water and the mixture heated for 20 min in a boiling water bath to remove soluble materials, which were washed away with excess distilled water.

The washed CLM from which soluble materials had been removed was suspended in 200 ml of 80% ethanol to remove water. The unreacted ECH was removed by washing the CLM with 200 ml of 98% ethanol. The CLM was washed with 100 ml of acetone and finally with 100 ml of petroleum ether. The CLM was allowed to stand for at least 15 min and then filtered under vacuum on a medium-fine (G3) sintered-glass filter at every washing step. The CLM was dried at room temperature (Inoue *et al.*, 1984) and ground to fine powder using a laboratory mill. The ground CLM was sifted using sieve no. 80 which has an aperture opening of 180 μm.

Homogeneous crosslinking of mucilage

A solution of crude mucilage (100 ml) was mixed with 40% DMSO (100 ml), ECH (40 ml) and 5 M NaOH (50 ml) in a stoppered 500 ml Erlenmeyer flask (Hatanaka *et al.*, 1990).

Purification of PG

Effect of pH on the binding of PG to CLM

The CLM, prepared by the standard method, was converted to the H^+ form by incubating overnight in 1 M HCl solution (Vogel, 1961). The CLM was washed with water until a neutral pH was obtained using litmus paper. The CLM in the H^+ form was then used for the purification of tomato PG.

To the dialysed solution of enzyme (2 ml) at pHs from 2.7 to 5.0 was added 1 g of swollen CLM previously equilibrated with McIlvaine buffer (0.025 M citrate–0.05 M sodium phosphate) at the same pH as that of the solution of enzyme. The suspension was incubated in a refrigerator at 4°C for 60 min with occasional stirring. After centrifugation at 3000 rpm in a bench centrifuge for 3 min, enzyme activities in the supernatants were measured (Inoue *et al.*, 1984).

Effect of volume of applied enzyme on the binding of PG to CLM

The pellets of swollen CLM (1 g) were resuspended in 8 ml of crude enzyme solution. The solution of enzyme was prepared by mixing the binding buffer (0.025 M citrate–0.05 M sodium phosphate at pH 4) with different volumes of crude enzyme at pH 4, making up all the volumes to 8 ml. The mixture of 8 ml of solution of enzyme and 1 g of CLM was allowed to stand for 60 min at 4°C with occasional stirring. The suspension was centrifuged at 3000 rpm in a bench centrifuge for 3 min. The PG activity and protein content in the supernatants were determined.

PG was routinely bound to CLM by incubating 1 g of CLM, pH 4, in 6 ml of solution of crude enzyme, pH 4, and 2 ml of 0.025 M citrate–0.05 M sodium phosphate, pH 4, for 60 min in a refrigerator at 4°C with occasional stirring.

After centrifugation, the CLM with bound enzyme was washed three times with McIlvaine buffer (0.06 M citrate–0.12 M phosphate buffer, pH 4).

In order to elute the adsorbed enzyme, the CLM–enzyme complex was incubated in 10 ml of 0.5 M NaCl in 0.06 M citrate–0.12 M phosphate buffer at pH 4 for 60 min at 4°C with occasional stirring. The suspension was centrifuged and the elution repeated with 10 ml of 1 M NaCl at pH 4. During washing and elution, enzyme activity and protein content in the supernatants were determined. The supernatants collected on elution and the solution of the crude enzyme were concentrated by freeze-drying and redissolving the samples in distilled water.

Regeneration and reuse of used CLM

After eluting the enzyme from the CLM, the used gel was regenerated to the H^+ form by incubating in 1 M NaCl overnight and washing in 1 M HCl several times. The CLM was washed with water to a neutral pH. The regenerated CLM was then used to purify tomato PG.

Determination of molecular weight of PG

The purification of PG was also assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12.5% gel according to the method of Bollag & Edelstein (1991). Before electrophoresis, the solutions of protein were heated for 2 min in the presence of 60 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol and 14.4 mM 2-mercaptoethanol. The gels were stained with 0.1% Coomassie Blue G-250 and destained with 7% acetic add. The molecular weight of the purified PG was estimated using protein standards from Promega and Sigma.

RESULTS AND DISCUSSION

Inadequate homogenization of the tomato fruit would result in failure to release the enzyme from the cell membrane and low recovery of activity. In order to release as much enzyme as possible, the pulp was homogenized in the presence of acid-washed sand in buffer. As shown in Fig. 1, the optimum pH for PG activity was around 4.

At pH 3 and below, all the protein was bound to CLM; at higher pHs a smaller proportion of enzyme was bound as shown in Table 1. At pH 2.7, binding was such that no protein was detected in the supernatant. It

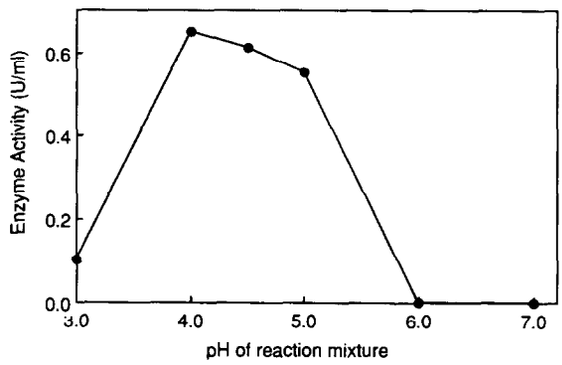

Fig. 1. Effect of pH on enzyme activity of PG extracted from tomato fruit. The pH of the solution of the crude enzyme was adjusted to the pHs indicated with 2 M buffer constituents. The activity of the enzyme of each solution was determined after incubation at 37°C for 30 min. The reducing groups were determined using the DNSA method.

Table 1. Effect of pH on binding of polygalacturonase (PG) to crosslinked mucilage (CLM)

pH of swollen CLM	Total protein applied (mg)	Total activity applied (units)	Total protein in supernatant (mg)	Total activity in supernatant (units)	Proportion of enzyme bound[a] (%)
2.7	2.52	2.1	0.0	0.0	100
3.0	1.17	2.34	0.23	0.0	100
4.0	5.19	2.52	0.66	0.32	87
4.5	2.21	2.22	0.22	0.24	89
5.0	5.26	2.4	0.29	0.56	77

[a]Proportion of enzyme bound when 6 ml of solution of enzyme at pH 2.7–5.0 was incubated with 1 g CLM is expressed as a percentage of the total amount of enzyme applied.

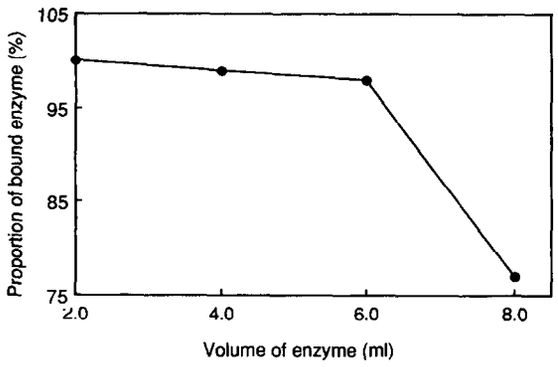

Fig. 2. Effect of volume of enzyme on the binding of PG to CLM. PG activity remaining in the supernatant when different volumes of enzyme were incubated with 1 g of CLM is expressed as a fraction of the total amount of enzyme applied. In all cases the final volume of enzyme was made up to 8 ml with buffer.

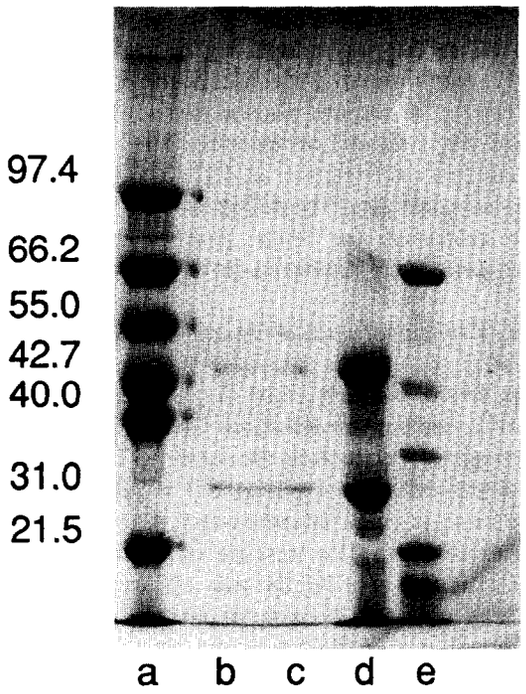

Fig. 3. SDS–PAGE of PG from tomato. Lane a, protein standards from Promega; lanes b and c, purified PG; lane d, crude enzyme extract; lane e, protein standards from Sigma.

is likely that, under these conditions, non-selective binding of protein by CLM occurred. At pH 4, low enzyme activity associated with a considerable amount of protein was detected in the supernatant. Under these conditions, it appeared that most of the enzyme was bound but a lot of contaminating protein remained in the supernatant. It was therefore decided to bind PG to the CLM at pH 4 in all the subsequent work.

When 1 g of CLM was loaded with increasing volume, most of the enzyme was bound, up to a loading level of 6 ml. After this, the proportion of bound enzyme decreased rapidly, as shown in Fig. 2. A loading volume of 6 ml enzyme g^{-1} CLM was considered to provide an acceptable balance between the volume of crude enzyme that was loaded and the proportion of enzyme that was bound and was adopted for the routine binding procedures.

The purification of PG from tomato fruit is summarized in Tables 2 and 3. Although the seven-fold purification that was achieved is modest, this was achieved in a single step. The level of purification compares favourably with the 8- to 42-fold purification that was achieved by Ali & Brady (1982) in a procedure involving up to five steps. One of the advantages of using water-insoluble matrices for the purification of enzymes is that the matrices are amenable to reuse after suitable treatment. It can be seen that regenerated used CLM was as effective in the purification of PG as the original matrix. It will be noted that the overall yield of enzyme during the purification procedure appears to exceed 100%. This apparent increased yield could arise if, during the purification process, factors that inhibit enzyme activity were removed.

Two main bands of protein with molecular weights of 30 000 and 44 000, respectively, remained after treatment

Table 2. Summary of purification of polygalacturonase (PG) from tomato using purified crosslinked mucilage (CLM)

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	4.0	5.2	0.77	1	100
0.5 M NaCl	3.4	0.63	5.4	7	85
1.0 M NaCl	2.3	0.46	5.0	6	58

The total amount of PG obtained when 1 g of CLM was eluted with NaCl was expressed as the fraction of the enzyme applied.

Table 3. Summary of purification of polygalacturonase (PG) from tomato using regenerated crosslinked mucilage (CLM)

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	4.5	6.47	0.69	1	100
0.5 M NaCl	4.8	1.2	4.0	6	107
1.0 M NaCl	2.5	0.6	4.2	6	56

The total amount of PG obtained when 1 g of CLM was eluted with NaCl was expressed as the fraction of the enzyme applied.

with fresh CLM, as shown in Fig. 3. The band at 44 000 corresponds to the molecular weight of tomato PG that has been reported by other workers (Pressey & Avants, 1973; Turker *et al.*, 1980; Bruinsma *et al.*, 1989). It appears that the band at 30 000 molecular weight is another pectin-recognizing enzyme that is not necessarily PG. The pattern of bands for regenerated CLM was similar.

CONCLUSION

It has been demonstrated that crosslinked ruredzo mucilage may be used for the purification of tomato PG. Used CLM can be regenerated and can be effectively used for the purification procedure.

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