

Pectate Hydrolases of Parsley (*Petroselinum crispum*) Roots

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The presence of various enzyme forms with terminal action pattern on pectate was evaluated in a protein mixture obtained from parsley roots. Enzymes found in the soluble fraction of roots (juice) were purified to homogeneity according to SDS-PAGE, partially separated by preparative isoelectric focusing and characterized. Three forms with pH optima 3.6, 4.2 and 4.6 clearly preferred substrates with a lower degree of polymerization (oligogalacturonates) while the form with pH optimum 5.2 was a typical exopolygalacturonase [EC 3.2.1.67] with relatively fast cleavage of polymeric substrate. The forms with pH optima 3.6, 4.2 and 5.2 were released from the pulp, too. The form from the pulp with pH optimum 4.6 preferred higher oligogalacturonates and was not described in plants previously. The production of individual forms in roots was compared with that produced by root cells cultivated on solid medium and in liquid one.

Key words: Exopolygalacturonase, Oligogalacturonate Hydrolase, Cell Suspension

Introduction

Exopolygalacturonases [poly(1,4- α -D-galacturonate)galacturonohydrolase, EC 3.2.1.67] are exohydrolases catalyzing the hydrolytic cleavage of glycosidic α -1,4-bonds of D-galacturonan at its nonreducing end releasing D-galactopyranuronic acid as the only reaction product. Substrates for these enzymes are polygalacturonic and oligogalacturonic acids and, in contrast to polygalacturonases [EC 3.2.1.15], also di-D-(galactosiduronic) acid (Rexová-Benková and Markovič, 1976). The particular enzymes differ from each other by the range and rate of the effects on substrate in relation to the chain length. It was supposed that unlike exopolygalacturonases of microbial origin, which favourize oligomers of lower DP (degree of polymerization), di-D-(galactosiduronic) acid or those, in which the substrate chain length is not a factor determining the enzyme effect, the most suitable substrates for exopolygalacturonases of plant origin are polymeric D-galacturonan or a partly degraded D-galacturonan of DP about 20 (Heinrichová, 1977 and references cited therein).

The first description of a plant enzyme with preference for oligogalacturonides (oligogalacturonate hydrolase, OGH) appeared in 2005 (Stratilová *et al.*, 2005), when an enzyme from carrot roots was described.

Although the first study of exopolygalacturonases from carrots (Hatanaka and Ozawa, 1964) indicated the presence of multiple forms of this enzyme based on the three pH optima present, later studies supported the idea of one form of exopolygalacturonase (Heinrichová, 1977; Pressey and Avants, 1975; Konno, 1988). In 1996 the multiple forms of exopolygalacturonase from carrot roots were identified (Stratilová *et al.*, 1996) and later the partial separation and characterization of four forms of this enzyme were described (Stratilová *et al.*, 1998). One of them, with pH optimum 3.8, clearly preferred oligomeric substrates. The found enzyme was the missing element of the enzyme chain needed for the complete hydrolysis of pectin in plants. On the other hand, the production of an enzyme with such a substrate preference is somewhat surprising, because of lack of enzyme

(polygalacturonase with random action pattern) in roots which could prepare the substrate for this oligogalacturonate hydrolase.

The function of pectate hydrolases with terminal action pattern or that of their product D-galactopyranuronic acid remains still unknown in plants. The objective of this work was to purify and characterize the pectate hydrolases from parsley roots as a new source. Enzymes obtained from roots were compared with those produced by callus and cell suspension cultures. The possibility to apply results obtained from cultivated root cells to natural roots was discussed.

Materials and Methods

Extraction of pectate hydrolases from parsley roots

Parsley roots (*Petroselinum crispum* cv. Olo-moucká dlouhá) in an amount of 20 kg from October crop were collected from the same field. They were treated immediately after their sampling. The first step of extraction of enzymes was performed on a juice extractor ES-3551 (Severin, Sundern, Germany). The obtained juice was filtered, precipitated with ammonium sulfate (Merck, Darmstadt, Germany) until a saturated solution was obtained as determined by refractometry (24 h, 4 °C), filtered again, dissolved in a small amount of water, dialyzed and freeze-dried. This process was followed by removing of low molecular substances on a Sephadex G-25 Medium column (Pharmacia, Sweden) (Rexová-Benková and Slezárik, 1966). The proteins retained in pulp were extracted 12 h with 0.1 M imidazole solution, pH 6.0, containing 1 M NaCl. After centrifugation (24,000 × g, 20 min, 4 °C), the obtained juice was handled as described previously.

Cultivation of cells from parsley roots on solid and liquid medium

Parsley root surface was sterilized in 10% Savox and washed 3 times in 3 × 500 ml sterile tap water for 30 min. Roots were cut into 1 mm thick transverse slices and transferred to a sterile Erlenmeyer flask (100 ml) containing Murashige and Skoog (MS) medium (vitamins, buffer including) with 3% sucrose, 1 mg/l 2,4-D and agar. These explants were incubated at room temperature in the dark until callus formation. After ca. 21 d the best-callusing explants were selected and, after cutting the callusing regions, transferred into fresh medium.

The friable, light-grown callus cultures were aseptically transferred into Erlenmeyer flasks (250 ml) containing the same MS buffer medium (without agar), and suspension cells were maintained under shaking and subcultured at a 7-days interval. The release of proteins from disrupted cells was provided with 0.1 M acetate buffer, pH 5.0, with 1 M NaCl. After centrifugation (24,000 × g, 20 min, 4 °C), the obtained juice was handled as described for extraction from roots.

Purification of pectate hydrolases

Pectate hydrolases from parsley juice were purified using gel-permeation and affinity chromatographies. The desalted protein mixture was applied on a Sephadex G-100 (Pharmacia) column (length 1.2 m, diameter 20 mm) in 0.1 M acetate buffer, pH 5.2 (flow rate 8 ml/h and 4 ml/fraction). Fractions with polygalacturonase activity were collected, desalted by dialysis and freeze-dried. The next step was provided on a concanavalin A-HEMA 1000 E column (length 25 mm, diameter 15 mm) in 0.1 M acetate buffer, pH 4.7, with addition of 0.1 M NaCl, 0.001 M MnCl₂ and 0.001 M CaCl₂ (fractions of 1 ml each). Concanavalin A-HEMA 1000 E was prepared by the reaction of concanavalin A (Fluka, Germany) with the epoxy groups of HEMA 1000 (Tessek, Praha, Czech Republic) in 0.1 M acetate buffer, pH 3.8 (11 d, 4 °C). 0.1 M Methyl- α -D-mannopyranoside (Sigma, Germany) in 0.1 M acetate buffer, pH 4.7, with 0.1 M NaCl was used as an eluting agent. FPLC was performed on Superose 12TM HR 10/30 (Pharmacia, Sweden) in 0.05 M phosphate buffer, pH 5.6, 0.15 M NaCl. The flow rate was 0.5 ml/min.

The partial separation of individual forms was performed utilizing preparative isoelectric focusing (IEF) in the pH region 3–7. After IEF the gel was divided in segments with a diameter smaller than 2 mm. The proteins were then washed out from these segments by water and the forms of pectate hydrolases were detected on the basis of their activities utilizing their pH optima and the substrates of preference.

Substrates

Commercial citrus pectin (Genu Pectin, Copenhagen, Denmark) was purified by washing with acidified 60% ethanol (5 ml conc. HCl/100 ml of 60% ethanol), followed by 60% and 96% neutral ethanol as described previously (Kohn and Furda,

1967). Pectate (DP 152) was prepared from this pectin by total alkaline deesterification (Kohn and Furda, 1967).

Oligogalacturonic acids (DP 2–10) were prepared by enzymatic hydrolysis of pectate (Heinrichová, 1983), gel filtration on a Sephadex G-25 Fine column in 0.05 M phosphate buffer, pH 7.0, and desalting on a Sephadex G-15 column (Rexová-Benková, 1970).

Unsaturated oligogalacturonic acids (DP 2, 3) were obtained after enzymatic degradation of potassium pectate by bacterial pectate lyase (1% pectate treated by lyase of *Erwinia* sp., pH 8.0, sustained by addition of 0.1 M KOH, 30 °C, 20 h) and freeze-drying.

Activity assay

Pectate hydrolase activity was assayed in 0.1 M acetate buffer at a pH value corresponding to the pH optimum of the individual form (pH optimum was evaluated in the range of pH 3.6–5.6) at 30 °C (except the determination of temperature optima at temperatures from 20 °C to 70 °C) by measuring the increase of colour intensity at 530 nm (Somogyi, 1952) in the reaction mixture containing solutions of substrates (1 mM solution of oligogalacturonates or 0.5% solution of sodium pectate). The enzyme activity was then expressed as the ratio of the increase in colour intensity of the respective fraction and of that with maximum colour intensity per time unit.

The thermal stability of enzymes was evaluated after 2 h of incubation of enzyme solutions at 20 °C–70 °C followed by the enzyme assay at 30 °C.

Characterization of pectate hydrolases

Individual pectate hydrolases were identified and characterized on the basis of differences in their pH optimum and initial rates on substrates with various DP. Michaelis parameters of enzymes purified from parsley juice were determined utilizing the initial velocities at five concentrations of substrate, ranging from 0.05 to 0.5 mM (substrates with DP 2, 5 and 7–8 mixture) or 0.05 to 0.25% (pectate), at 30 °C, and calculated by nonlinear regression.

The products of hydrolysis of pectate or natural and unsaturated oligogalacturonates were analyzed by thin-layer chromatography on Silica gel 60 sheets (Merck) using *n*-butanol/formic acid/wa-

ter (2:3:1) as the eluent (Koller and Neukom, 1964). The spots were detected by 20% (w/v) solution of ammonium sulfate in water, D-galactopyranuronic acid being the reference substance.

For deglycosylation of enzymes purified from parsley juice, *N*-glycosidase A (Roche Diagnostics, Mannheim, Germany) was used. The cleavage of pectate hydrolases denaturated by a boiling water bath (10 min) in the presence of 5% mercaptoethanol and 1% SDS was performed as recommended [0.1 M phosphate buffer, pH 7.2, containing 0.1% SDS, 1% mercaptoethanol, 0.025 M EDTA, and 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)]. The cleavage was performed for 24 h at 37 °C. The change of the relative molecular mass of pectate hydrolases was detected by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

SDS-PAGE for molecular mass analysis of native and deglycosylated pectate hydrolases was performed on a Mini-Protean 3 Electrophoresis System (Bio-Rad Laboratories, Hercules, California) under reducing conditions (with β -mercaptoethanol). The silver-staining method was used for band visualization (Wray *et al.*, 1981). The following standard calibration proteins in the range 17–95 kDa (Serva) were utilized: Phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Ultrathin-layer isoelectric focusing in polyacrylamide gels on polyester films was performed as previously described by Radola (1980). Standards (protein test mixture for pI-determination, pH 3–10, Serva, Heidelberg, Germany) were stained with Serva Violet 49. Activities of pectate hydrolases were determined by the zymogram technique with a colourless D-galacturonan DP 10 followed by staining of noncleaved substrate with ruthenium red.

N-Terminal sequencing of SDS-homogeneous protein was provided in Procise – protein sequencing system (Applied Biosystems, Foster City, CA, USA).

Results and Discussion

The protein precipitates from parsley roots, callus and cell suspension were examined for the presence of pectate hydrolases on the basis of their activity on pectate and penta-D-(galactosidu-

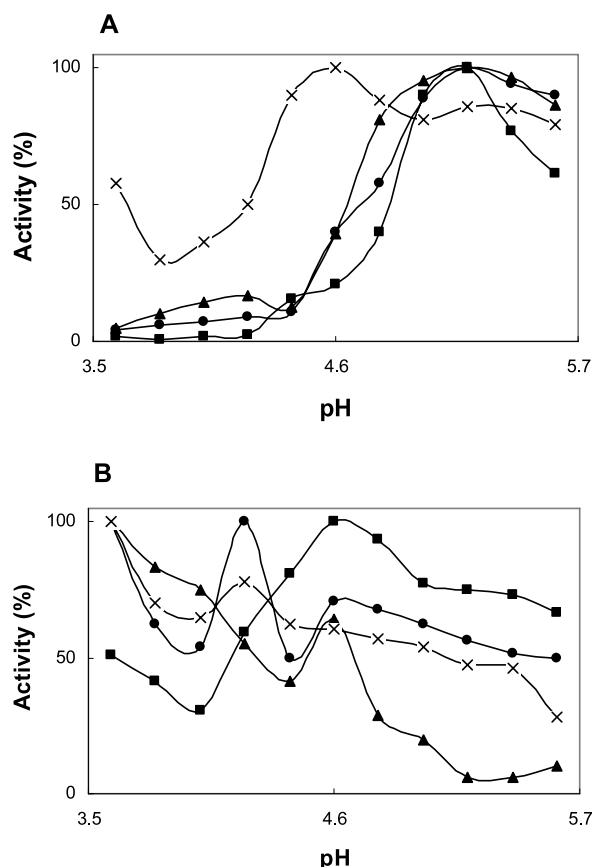


Fig. 1. pH optimum of pectate hydrolases in parsley: A, Activity on 0.5% sodium pectate; B, activity on 1 mM pentagalacturonate. ● – roots juice; x – roots pulp; ■ – callus; ▲ – cell suspension.

ronic) acid at various pH values (Fig. 1) as described for enzymes from carrot roots (Stratilová *et al.*, 1998). One form of exopolysaccharuronase and even more forms of oligogalacturonate hydrolases than in carrots were found in parsley showing a wide variety of exo-enzymes with the ability to completely degrade parts of pectate in wall pectin. The common exo-manner of all present forms was evaluated by TLC of the degradation products (D-galactopyranuronic acid as a sole product) and by correlation of viscosity decrease with the degree of polymer degradation (degradation of 10% of pectate bonds caused only 15% viscosity decrease). The orientation of substrate splitting was evaluated using substrates modified by endopectate lyase. Reaction of these unsaturated oligogalacturonates (DP 2, 3 marked on the nonreducing end by the double bond) with pectate hydrolases

from parsley did not lead to any splitting of these modified substrates, *e.g.* these enzymes utilized the cleavage of substrates from their nonreducing end as was described for enzymes from carrot roots (Heinrichová, 1977; Stratilová *et al.*, 2005).

Pectate hydrolases with terminal action pattern preferring oligomeric substrates were described as typical enzymes produced by various microorganisms (Rexová-Benková and Markovič, 1976). To prevent misinterpretation of results obtained with roots cropped from the field (possibility of contamination) sterile cell cultures from these roots growing on solid and liquid medium were prepared. The comparison of the occurrence of pectate hydrolases in roots, callus and cell suspension showed that the same enzyme forms were present but their ratio varied considerably (Fig. 1) probably due to various stages of plant growth and different conditions. Accordingly, the usage of callus or cell suspension for comparison of all produced forms or the study of the sense of their production in roots can lead to incorrect conclusions. This was indicated for exopolysaccharuronases from carrot roots where other forms for cell suspension (Konno *et al.*, 1989) and roots (Stratilová *et al.*, 1998, 2005) were described. On the other hand the protein extracts obtained from cell cultures can be useful for purification and characterization of individual enzyme forms.

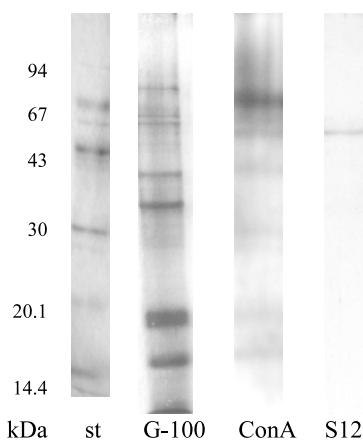


Fig. 2. SDS-PAGE of purified pectate hydrolases from parsley roots juice after each purification step: G-100, Sephadex G-100 step; ConA, proteins after concanavalin A-HEMA 1000 E step; S12, Superose 12 step. The silver-staining method was used for band visualization. Standard calibration proteins in the range 14.4–94 kDa (Serva) were utilized: Phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa).

Table I. Characterization of pectate hydrolases from parsley roots.

pH optimum	Occurrence	DP of substrate of preference	Enzyme	Approx. Ip
About 3.6	Roots (juice/pulp), callus, cells	5–7	OGH	5.45
About 4.2	Roots (juice/pulp)	5–7	OGH	5.35
About 4.6	Roots (juice), callus, cells	5–7	OGH	5.6
About 4.6	Roots (pulp)	10	OGH	nd
About 5.2	Roots (juice/pulp), cells	PGA	exoPG	5.55
About 5.2	Callus	10	OGH	nd

Juice/pulp, soluble fraction of roots/solid fraction of roots (enzymes released with 1 M NaCl).
nd, not determined.

The purification procedure of pectate hydrolases from the protein precipitate of juice from parsley roots was monitored after each chromatography step (Sephadex G-100, concanavalin A-HEMA 1000 E and Superose 12) by SDS-PAGE (Fig. 2). Unfortunately the protein with the molecular mass about 55.3 kDa and apparently homogeneous by SDS-PAGE included still a mixture of proteins as was shown by *N*-terminal analysis. Alanine and threonine seemed to be the *N*-terminal amino acids of the major forms of pectate hydrolases.

The affinity of all produced forms towards concanavalin A indicated the presence of similar or identical glycosylation. This was confirmed by *N*-glycosidase A cleavage. As was shown by SDS-PAGE, only about 1 kDa decrease was observed after deglycosylation of denaturated proteins.

The separation of individual pectate hydrolases was performed by preparative IEF. The enzymes

washed out from gel segments were identified on the basis of their pH optima and substrate preference (Fig. 3). The isoelectric points of enzymes were then evaluated by IEF utilizing IEF standards and the zymogram technique for the localization of enzymes in the gel. The isoelectric points were very close together, between pH 5.3 and 5.6 (Table I).

At least six pectate hydrolases with pH optima 3.6, 4.2, 4.6 (two different forms) and 5.2 (two different enzymes) were produced in parsley roots (Fig. 1, Table I). The comparison of these forms from the point of view of their substrate preference is given in Fig. 4. The enzymes with more acidic pH optima, 3.6, 4.2 and 4.6, as well as the enzyme from callus with pH optimum 5.2 can be supposed to be oligogalacturonate hydrolases (OGHs). The enzyme occurring both in original roots and cell suspension with pH optimum 5.2 is a typical exopolygalacturonase (Figs. 1, 4, Table I). The comparison of initial rates of individual en-

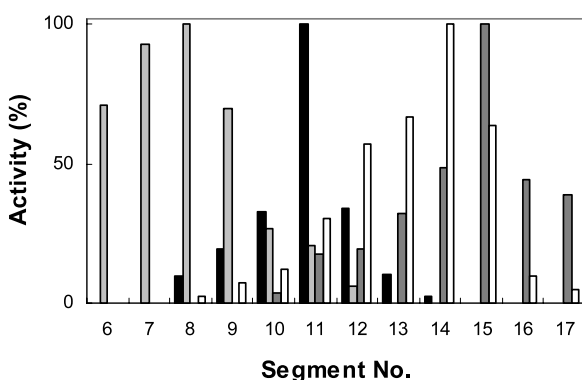


Fig. 3. Preparative IEF of proteins from parsley roots juice with M_r 55.3 kDa. Activities of pectate hydrolases on substrates: ■ – 1 mM pentagalacturonate, pH 3.6; ■ – 1 mM pentagalacturonate, pH 4.2; ■ – 1 mM pentagalacturonate, pH 4.6; □ – 0.5% pectate, pH 5.2.

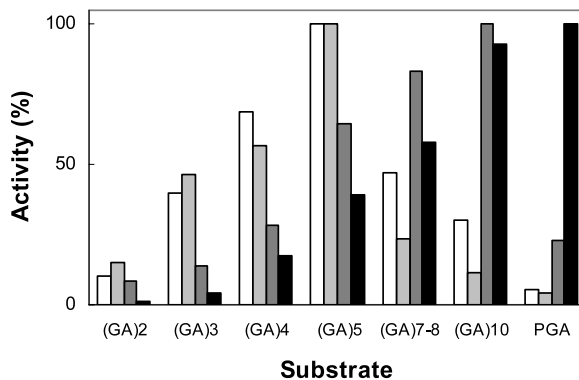


Fig. 4. The initial rates of pectate hydrolases from parsley roots pulp on substrates with various DP: enzyme with pH optimum 3.6 – □, 4.2 – ■, 4.6 – ■ and 5.2 – ■.

zymes on substrates with various DPs in Fig. 4 is demonstrated with enzymes obtained from the pulp because of the absence of OGHs with preference for higher oligosubstrates in the roots juice. This enzyme with pH optimum 4.6 found in the pulp of roots or with pH optimum 5.2 found in callus (Table I) was the most surprising one, a pectate hydrolase preferring higher oligogalacturonates as substrates (Fig. 4).

The enzymes detected in the juice of roots were further characterized.

The temperature optimum of all forms was between 60 and 70 °C. They were still stable by 55 °C (100% recovery of activity after 2 h). For comparison, the temperature optimum of the major form of exopolygalacturonase (Heinrichová, 1977) and OGH (Stratilová *et al.*, 2005) from carrot roots were in the same region. Both enzymes showed very high temperature stabilities, too.

In general, exopolygalacturonase from carrot roots with pH optimum 5.0 is very similar to exopolygalacturonase from parsley roots with pH optimum 5.2, and OGH from carrots with pH optimum 3.8 with them from parsley with pH optima 3.6, 4.2 and one form with pH optimum 4.6. This similarity indicating the same function in roots of both plants was supported by results obtained from kinetic analyses provided on di-D-(galactosiduronic) acid, penta-D-(galactosiduronic) acid

and pectate. As expected from results for carrot enzymes (Stratilová *et al.*, 2005), the affinity of both enzymes, OGH and exopolygalacturonase, increased with increasing DP of the substrate (decrease of K_M with DP increase), but the increase of the maximal rate was stopped by OGH when polymeric substrate was used. The initial rates on substrates with various DPs indicated that these maxima of the maximal rate were reached for DP 6 as it was for carrot OGH (Stratilová *et al.*, 2005) or DP 10 (Fig. 4). The form of pectate hydrolase found in the pulp and callus which favoured decagalacturonate as a substrate was not found yet in carrots or other plants.

Results of this work will serve as a basis for structural evaluation of individual pectate hydrolases as well as for structure-function studies.

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