Oligogalacturonate Hydrolase from Carrot Roots

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Z. Naturforsch. **60 c**, 899–905 (2005); received April 27/May 24, 2005

The presence of multiple forms of enzyme with terminal action pattern on pectate was evaluated in the protein mixture obtained from carrot roots. The form with pH optimum 3.8 clearly preferred substrates with a lower degree of polymerization (oligogalacturonates). Its molecular mass, isoelectric point, glycosylation as well as cleavage of pectate from nonreducing end corresponded to an exopolygalacturonase [EC 3.2.2.67]. The affinity of this enzyme to the substrates increased with the increasing degree of polymerization, and the difference was observed only in the maximal ratio of catalysis of oligomeric and polymeric substrates. Sterical hindrance for substrates with more than six D-galactopyranuronic acid units is supposed and an oligogalacturonate hydrolase rather than exopolygalacturonase is considered.

Key words: Daucus carota, Exopolygalacturonase, Oligogalacturonate Hydrolase

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 under releasing D-galactopyranuronic acid as the sole 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 particullar 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).

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 present pH optima, 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.*, 1996a) and later the partial separation and characterization of four forms of this enzyme was described (Stratilová *et al.*, 1998). One of them, with pH optimum 3.8, clearly preferred oligomeric substrates.

The aim of this work is to characterize this enzyme. The found enzyme is the missing element of the enzyme chain needed for complete hydrolysis of pectin in plants. We suggest the denotation "oligogalacturonate hydrolase" rather than exopolygalacturonase.

Materials and Methods

Extraction of exopolygalacturonases from carrot roots

Carrots (*Daucus carota* cv. Tiptop) in an amount of 50 kg of roots from September crop were collected from the same field. The carrots 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 precipitated with ammonium sulfate (Merck, Germany) until a saturated solution was obtained as determined by refractometry (24 h, 4 °C), filtered, dissolved in a small amount of water and precipitated with 96% ethanol (1:4 v/v, 24 h, 4 °C) (Rexová-Benková and Slezárik, 1966) followed by dialysis, freeze-drying,

and removing of low molecular substances on a Sephadex G-25 Medium column (Pharmacia, Sweden).

Purification of oligogalacturonate hydrolase

The individual forms of pectate hydrolases from carrot juice were purified from the protein mixture using ion-exchange, affinity and gel-permeation chromatographies as described by Stratilová *et al.* (1996a). For CM-Sephadex C-50 chromatography (Pharmacia, Sweden) stepwise elution was used with 0.05 M acetate buffer, pH 3.8, 0.10 M acetate buffer, pH 4.8, 0.15 M acetate buffer, pH 5.6, and, finally, this buffer containing 1.0 M NaCl. The proteins with exopolygalacturonase activity eluted with 0.15 M acetate buffer, pH 5.6, in the first peak (Stratilová *et al.*, 1996a) were after desalting and freeze-drying applied on a concanavalin A-cellulose column (Mislovičová *et al.*, 1995) in 0.1 M acetate buffer, pH 4.7. 0.1 M Methyl-α-D-mannopyranoside (Sigma, Germany) was used as an eluting agent. FPLC (Pharmacia, Sweden) was performed on a Superose 12™ HR 10/30 column in 0.05 M phosphate buffer, pH 5.6, 0.15 M NaCl. The flow rate was 0.5 ml/min. The separation of exopolygalacturonase and oligogalacturonate hydrolase was performed on a Phenyl Superose column HR 5/5 (FPLC, Pharmacia, Sweden) utilizing a linear gradient of 0.05 M phosphate buffer, pH 7.0, with 1.7 M ammonium sulfate to 0.05 M phosphate buffer, pH 7.0. The flow rate was 0.5 ml/min. The rest of proteins was removed from the column by 200 μl of 99% ethylene glycol by decreased flow rate (0.2 ml/min). The activity assay was made after desalting of fractions on PD 10 (Pharmacia, Sweden).

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 with DP 2–7 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. Reduced oligogalacturonates were prepared from the oligogalacturonates (DP 2, 3) by treating with sodium borohydride according to McCready and Seegmiller (1954). In this way the reduced galacturonic acid unit was changed to galactonolactone. Following the reduction step the products were desalted by gel filtration on Sephadex G-10 as described (Rexová-Benková, 1973).

Activity assay

Exopolygalacturonase activity was assayed in 0.1 M acetate buffer, pH values corresponding to the pH optimum of the individual form (pH 3.8 and pH 5.0; Stratilová *et al.*, 1998) at 30 °C (except the determination of temperature optima at temperatures from 20 °C to 70 °C) by measuring the increase of color 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 expressed in μmol reducing groups (using a standard graph for D-galactopyranuronic acid) liberated within 1 min by 1 mg protein. The activity of fractions eluted from columns was simply expressed as an increase of absorbance at 530 nm under standard conditions (solution of pectate or pentagalacturonate, pH optimum of enzyme, 30 °C and identical time interval for enzyme-substrate incubation).

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

Characterization of oligogalacturonate hydrolase

Michaelis parameters were determined utilizing the initial velocities at five concentrations of substrate, ranging from 0.05 to 0.5 mM (substrates with DP 2–7) or 0.05 to 0.25% (pectate), 30 °C, and calculated by nonlinear regression. Affinities of subsites were calculated according to Hiromi *et al.* (1973). The suggested active site was then compared with the active site of the main form of

carrot exopolygalacturonase (Heinrichová *et al.*, 1995).

The products of the hydrolysis of pectate or natural and unsaturated oligogalacturonates were analysed by thin-layer chromatography on Silufol silica sheets (Kavalier, Žďár nad Sázavou, Czech Republic) using *n*-butanol/formic acid/water (2:3:1) as the eluent (Koller and Neukom, 1964). The spots were detected by aniline phthalate reagent, D-galactopyranuronic acid being the reference substance. The reduced derivatives of oligogalacturonates were chromatographed on Whatman No. 4 in the solvent system ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). The reduced compounds behaved as nonreducing sugars and were detected as lactones with hydroxylamine reagent according to Gee and McCready (1957).

Approximate relative molecular masses (M_r) of enzymes were determined by gel filtration on a Superose 12^{TM} HR 10/30 column connected to a FPLC device in 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.0. Ferritin (M_r 450 kDa), catalase (240 kDa), aldolase (158 kDa), albumin (68 and 45 kDa), chymotrypsinogen A (25 kDa) and cytochrome C (12.5 kDa) were used as the standards ("Combithek" Calibration proteins II, Boehringer-Mannheim, Germany).

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

SDS-PAGE for molecular mass analysis of native and deglycosylated oligogalacturonate hydrolase 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). 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). Proteins were stained with Serva Violet 49 (Heidelberg, Germany). Exopolygalacturonase activities were determined by the zymogram technique with a dyed substrate (Ostazin Brilliant Red/D-galacturonan DP 10) (Markovič *et al.*, 1992) or with colourless D-galacturonan DP 10 followed by staining of not cleaved substrate with ruthenium red.

The exopolygalacturonase forms were washed out by water from frozen gel segments obtained by preparative isoelectric focusing. Activities of such separated forms were detected using solutions of pentagalacturonate (1mM in 0.1 M acetate buffer, pH 3.8) and pectate (0.5% in 0.1 M acetate buffer, pH 5.0).

Results and Discussion

The purification procedure of oligogalacturonate hydrolase was monitored after each chromatography step by gel-permeation chromatography on a Superose 12^{TM} column (Stratilová *et al.*, 1996a, b). SDS-PAGE was not suitable for this purpose because of irreversible inactivation of enzyme in running buffer as well as because of low protein content of enzyme in comparison with contaminants in the crude extract from carrot roots.

Two protein peaks with exopolygalacturonase activity were obtained by the elution of crude precipitate from carrot juice on a CM-Sephadex C-50 column with 0.15 M acetate buffer, pH 5.6 (Stratilová *et al.*, 1996a). The pH optimum determination on pectate of the first one showed the presence of two enzymes: exopolygalacturonase with pH optimum 5.0, described and relatively well characterized earlier (Heinrichová, 1977; Heinrichová and Perečková, 1983; Heinrichová *et al.*, 1995), and an apparent minor enzyme form with pH optimum 3.8. The determination of pH optimum on penta-D-(galactosiduronic) acid compared with a previous one shows clearly the preference of substrates with a lower degree of polymerization for this enzyme (Fig. 1).

The attempt to separate these two enzyme forms on a concanavalin A-bead cellulose column (Stratilová *et al.*, 1996b) was not successful. A

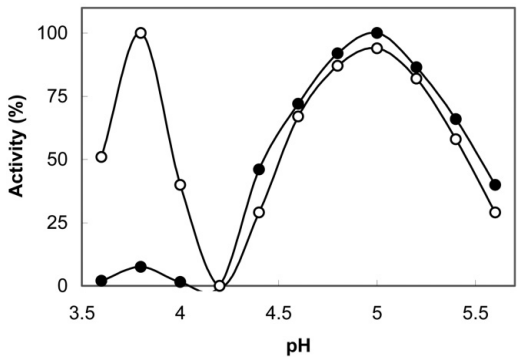

Fig. 1. pH optimum of exopolygalacturonase and oligogalacturonate hydrolase in carrot juice; (●–●) – activity on 0.5% sodium pectate; (○–○) – activity on 1 mM pentagalacturonate.

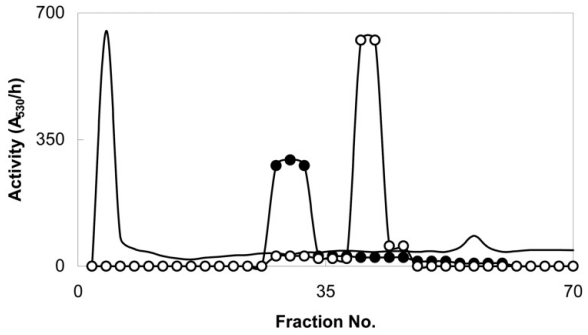

Fig. 2. The separation of exopolygalacturonase and oligogalacturonate hydrolase from carrot roots on a Phenyl Superose column (FPLC). Activity is expressed as A_{530}; (●–●) – 1 mM pentagalacturonate, pH 5.0 and (○–○) – 1 mM pentagalacturonate, pH 3.8 were used. Proteins are expressed as A_{280} (–). Conditions: Linear gradient of 0.05 M phosphate buffer, pH 7.0, with 1.7 M ammonium sulfate to 0.05 M phosphate buffer, pH 7.0; flow rate 0.5 ml/min.

huge amount of contaminants was removed (about fifty-fold purification) as in the case of ion-exchange chromatography (Stratilová *et al.*, 1996a), but the affinity of both enzymes to concanavalin A remained the same. The elution profile of this fraction on a Superose 12 column indicated the same retention time for activities of both enzymes, what made the separation on the basis of molecular weight unable (Stratilová *et al.*, 1996a).

As indicated by ion-exchange chromatography, thin-layer isoelectric focusing showed very similar isoelectric points of both enzymes (Stratilová *et al.*, 1996a). This method as well as chromatofocusing on a Mono P column (FPLC) was not suitable for separation of these forms, but the preparative isoelectric focusing with activity assay in both substrates of difference brought the evidence that really two enzyme forms were present in this fraction and not only one form with preference of substrates with various DP at different pH values (Stratilová *et al.*, 1998).

Separation of these two enzyme forms, a typical exopolygalacturonase and an enzyme for which the name oligogalacturonate hydrolase could be suggested, was performed utilizing hydrophobic interactions on a Phenyl Superose column (Fig. 2). The desalted fractions 40–42 were used for further characterization.

As showed by the purification process oligogalacturonate hydrolase is the enzyme form from carrot roots with pH optimum 3.8, molecular mass about 50 kDa (as determined by gel filtration) and isoelectric point about 4.5 (Stratilová *et al.*, 1998). Its glycosylation led to affinity towards concanavalin A (Stratilová *et al.*, 1996a) and was confirmed by *N*-glycosidase F cleavage (Fig. 3). SDS-PAGE showed a molecular weight of about 54 kDa decreasing after deglycosylation to 31.5 kDa.

The temperature optimum of oligogalacturonate hydrolase was almost 70 °C. The activity of oligo-

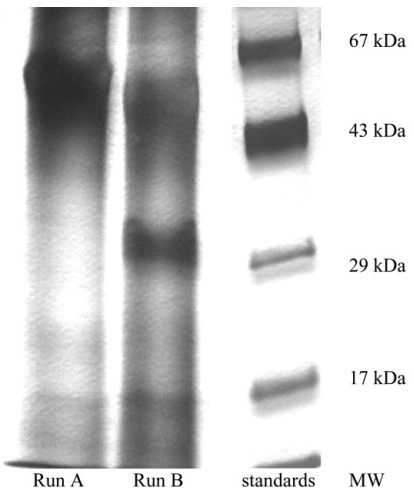

Fig. 3. SDS-PAGE of purified oligogalacturonate hydrolase from carrot roots. Run A, natural enzyme; run B, enzyme deglycosylated by *N*-glycosidase F. The silver-staining method was used for band visualization. Standard calibration proteins in the range 14–94 kDa (Serva) were utilized: Phosphorylase 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. Kinetic analysis of substrates degradation with DP 2–8 by oligogalacturonate hydrolase from carrot roots.

Substrate $(GA)_{DP}$*	K_M [mol/l]	V [μmol/min]	k_0 [1/s]	log k_0	k_0/K_M	log k_0/K_M
$(GA)_2$	8.6×10^{-4}	0.21	0.17	– 0.766	199.5	2.30
$(GA)_3$	1.9×10^{-4}	0.53	0.44	– 0.358	2269.4	3.36
$(GA)_4$	1.0×10^{-4}	0.63	0.52	– 0.283	5105.9	3.71
$(GA)_5$	7.7×10^{-5}	0.68	0.57	– 0.246	7422.2	3.87
$(GA)_6$	5.7×10^{-5}	0.70	0.59	– 0.232	10280.7	4.01
$(GA)_7$	2.9×10^{-5}	0.40	0.33	– 0.477	11695.9	4.07
$(GA)_8$	2.0×10^{-5}	0.37	0.31	– 0.515	14990.2	4.18
PGA	degraded extremely slowly, no possibility to provide the kinetic analysis					

* GA, D-galactopyranuronic acid unit.

galacturonate hydrolase after 2 h at 60 °C was about 90%, but at 70 °C it drastically decreased. For comparison, the temperature optimum and high stability of the major form of exopolygalacturonase from carrot roots was between 60 and 70 °C (Heinrichová, 1977).

The preference of low molecular substrates by this enzyme led to kinetic analysis of degradation of substrates with DP 2–8 (Table I). Surprisingly, the affinity of this enzyme increased with increasing DP of substrate as was determined for exopolygalacturonase (Heinrichová *et al.*, 1995). The difference between these two enzymes was observed in value of their maximal reaction rate, K_M, on substrates with different DP. While this value for exopolygalacturonase increased with increasing DP of substrate (Heinrichová *et al.*, 1995) for oligogalacturonate hydrolase it increased from DP 2 and it reached the maximum at DP 6 and then with further increase of DP decreased (Table I). This may explain the observation that oligogalacturonate hydrolase is able to degrade the dyed substrate Ostazin Brilliant Red/D-galacturonan DP 10 (Stratilová *et al.*, 1996a) which is undegradable by other carrot exopolygalacturonases. From this reason this dyed substrate is not suitable for distinguishing of pectate hydrolases with random and terminal action pattern as was supposed by Markovič *et al.* (1992).

The orientation of substrate splitting was evaluated using substrates modified by reduction and endopectate lyase. Degradation of oligogalacturonates (DP 2, 3) modified by reduction of the saccharide unit on the reducing end to lactone showed that the trimer was cleaved on monomer and marked dimer. In contrast with previous observations for exopolygalacturonase (Heinrichová, 1977) the dimer was degraded, too, but extremely slowly. Degradation of unsaturated oligogalacturonates (DP 2, 3) obtained after treatment of pectate with endopectate lyase (oligogalacturonate marked on nonreducing end by double bound), with oligogalacturonate hydrolase and exopolygalacturonase did not lead to any splitting of these modified substrates. This led to the conclusion that oligogalacturonate hydrolase utilizes cleavage of substrates from their nonreducing end as exopolygalacturonase (Heinrichová, 1977).

The obtained parameters (Table I) were used for the calculation of the subsite affinities according to the method described by Hiromi *et al.* (1973). The reason for the choice of this method was its use by Heinrichová *et al.* (1995) for calculation of the subsite affinities of carrot exopolygalacturonase. After calculation of the missing affinities of first and second subsites the comparison of the active site of exopolygalacturonase and oligogalacturonate hydrolase from the same source was performed (Table II).

The active sites of both enzymes contain six subsites (Fig. 4). The arrangement of subsite binding affinities is very similar with the highest affinity on subsite A_2. A_1 of exopolygalacturonase is higher than A_3 and A_4, in the case of oligogalacturonate hydrolase it is opposite (Table II). Such an ar-

Table II. The subsite affinities of exopolygalacturonase and oligogalacturonate hydrolase from carrot roots (catalytic site between first and second subsite).

Subsite [kJ/mol]	Oligogalacturonate hydrolase	Exopolygalacturonase*
A_1	1.75	2.41
A_2	29.56	32.58
A_3	6.13	1.53
A_4	2.04	0.59
A_5	0.94	0.35
A_6	0.82	0.27

* Subsite affinities of subsites 3–6 from Heinrichová *et al.* (1995).

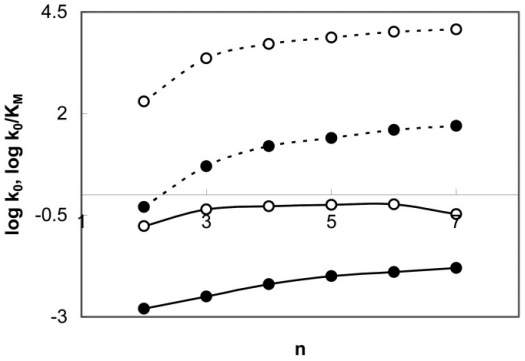

Fig. 4. The dependence of kinetic properties of oligogalacturonate hydrolase (○) and exopolygalacturonase (●, Heinrichová *et al.*, 1995) from carrot roots on DP. (–), log k_0; (---), log k_0/K_M.

rangement of active site of oligogalacturonate hydrolase can hardly explain the relatively high reaction rate on digalacturonate, but this problem may be answered in future structural studies.

Conclusion

The separation of exopolygalacturonase and oligogalacturonate hydrolase was reached only on the basis of their different hydrophobicity. Both enzymes have very similar properties, as terminal action pattern, cleavage of substrates from nonreducing end, affinity towards oligogalacturonates increasing with increasing DP of substrate (K_M decreased with increasing chain length of substrate), molecular masses, isoelectric points, glycosylation, thermal stability and temperature optimum. Enzymes differ in pH optima and in their values of maximal reaction rate in dependence on DP of oligosubstrates. The highest affinity on subsite A_2 was counted for both enzymes, but A_1 of exopolygalacturonase is higher than A_3 and A_4. In the case of oligogalacturonate hydrolase are A_3 and A_4 higher than A_1. Oligogalacturonate hydrolase degrades the dyed substrate Ostazin Brilliant Red/D-galacturonate DP 10 as enzymes with random action pattern do, but only because its hight reaction rate on oligomeric substrates.

Pressey and Avants (1975) discussed the unability of complete hydrolysis of pectin in plants (namely in carrots), because of decrease of reaction rate of exopolygalacturonases on oligomeric substrates. Oligogalacturonate hydrolase from carrot roots described in this work clarifies this problem. In plants producing this enzyme the hypothesis of García-Romera and Fry (1995) about lower oligogalacturonates as biologically inactive and therefore no more degraded cannot be valid.

Acknowledgements

The authors are grateful to Mrs. Helena Čigašová and Mr. T. Lipka for excellent technical assistance. This research was supported by the Slovak Grant Agency (VEGA Nos. 2/3160/23 and 2/3162/23) and by a grant (No. II/2/2005) from the Slovak Academy of Sciences to the Centre of Excellence GLYCOBIOS.

García-Romera I. and Fry S. C. (1995), The longevity of biologically-active oligogalacturonides in rose cell cultures. Degradation by exo-polygalacturonase. J. Exp. Botany **46**, 1853–1857.

Gee M. and McCready R. M. (1957), Paper chromatographic detection of galacturonic and glucuronic acid. Anal. Chem. **29**, 257–258.

Hatanaka C. and Ozawa J. (1964), Enzymic degradation of pectic acid. I. Limited hydrolysis of pectic acids by carrot exopolygalacturonase. Agr. Biol. Chem. **28**, 627–632.

Heinrichová K. (1977), Isolation, characterization and mode of action of exo-D-galacturonanase from carrot. Collect. Czech. Chem. Commun. **42**, 3214–3221.

Heinrichová K. (1983), Preparation of oligogalacturonic acids by enzymatic hydrolysis. Biologia, Bratislava **38**, 335–342.

Heinrichová K. and Perečková J. (1983), Multi-chain action of exo-D-galacturonanase from carrot. Collect. Czech. Chem. Commun. **48**, 3579–3588.

Heinrichová K., Heinrich J., and Dzúrová M. (1995), Partial characterization of the active site of carrot poly(galacturonate) hydrolase and degradation of oligo(D-galactosiduronates). Collect. Czech. Chem. Commun. **60**, 328–338.

Hiromi K., Nitta Y., Numata C., and Ono S. (1973), Subsite affinities of glucoamylase: Examination of the validity of the subsite theory. Biochem. Biophys. Acta **302**, 362–375.

Kohn R. and Furda I. (1967), Calcium ion activity in solutions of calcium pectinate. Collect. Czech. Chem. Commun. **32**, 1925–1937.

Koller A. and Neukom H. (1964), Detection of oligogalacturonic acids by thin-layer chromatography. Biochim. Biophys. Acta **83**, 366–367.

Konno H. (1988), Galacturonan 1,4-α-galacturonidase from carrot *Daucus carota* and liverworth *Marchantia polymorpha*. Methods Enzymol. **161**, 373–380.

Markovič O., Mislovičová D., Biely P., and Heinrichová K. (1992), Chromogenic substrate for *endo*-polygalacturonase detection in gels. J. Chromatogr. **603**, 243–246.

McCready R. M. and Seegmiller C. G. (1954), Action of pectic enzymes on oligogalacturonic acids and some of their derivates. Arch. Biochem. Biophys. **50**, 440–450.

Mislovičová D., Chudinová M., Gemeiner P., and Dočolomanský P. (1995), Affinity chromatography of invertase on concanavalin A-bead cellulose matrix: The case of extraordinary strong binding glycoenzyme. J. Chromatogr. B **664**, 145–153.

Pressey R. and Avants J. K. (1975), Modes of action of carrot and peach exopolygalacturonases. Phytochemistry **14**, 957–961.

Radola B. J. (1980), Ultrathin-layer isoelectric focusing in 50–100 μm polyacrylamide gels on silanized plates or polyester films. Electrophoresis **1**, 43–56.

Rexová-Benková Ľ. (1970), Separation of oligogalacturonic acids by dextran gel chromatography. Chem. Zvesti **24**, 59–62.

Rexová-Benková Ľ. (1973), The size of the substrate-binding site of an *Aspergillus niger* extracellular endopolygalacturonase. Eur. J. Biochem. **39**, 109–115.

Rexová-Benková Ľ. and Slezárik A. (1966), Isolation of extracellular pectolytic enzymes produced by *Aspergillus niger*. Collect. Czech. Chem. Commun. **31**, 122–129.

Rexová-Benková Ľ. and Markovič O. (1976), Pectic enzymes. Adv. Carbohydr. Chem. Biochem. **33**, 323–385.

Somogyi M. (1952), Notes on sugar determination. J. Biol. Chem. **195**, 19–23.

Stratilová E., Dzúrová M., and Mislovičová D. (1996a), Multiple forms of carrot exopolygalacturonase. In: Pectin and Pectinases, Progress in Biotechnology 14 (Visser J. and Voragen A. G. J., eds.). Elsevier Science, Amsterdam, pp. 807–814.

Stratilová E., Mislovičová D., and Dzúrová M. (1996b), Purification of exopolygalacturonase by affinity chromatography on concanavalin A-bead cellulose. Biotechnol. Tech. **10**, 363–366.

Stratilová E., Markovič O., Dzúrová M., Malovíková A., Capek P., and Omelková J. (1998), The pectolytic enzymes of carrots. Biologia, Bratislava **53**, 731–738.

Wray W., Boulikas T., Wray V. P., and Hancock R. (1981), Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**, 197–203.