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Purification and mechanistic characterisation of two polygalacturonases from *Sclerotium rolfsii*

W. Schnitzhofer [a], H.-J. Weber [b], M. Vršanská [c], P. Biely [c], A. Cavaco-Paulo [d], G.M. Guebitz [a,*]

[a] *Department of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria*
[b] *Department of Organic Chemistry, Graz University of Technology, Stremayrgasse 16, 8010 Graz, Austria*
[c] *Institute of Chemistry, Slovak Acadamy of Sciences, 84238 Bratislava, Slovak Republic*
[d] *Department of Textile Engineering, University of Minho, 4800 Guimaraes, Portugal*

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Abstract

Sclerotium rolfsii (strain CBS 350.80) was found to produce extraordinary high amounts of polygalacturonases (PGs). Two of these extracellular enzymes were purified by a recently introduced preparative electrophoretic device (isoelectric focusing mode of free flow electrophoresis). PG 1 (39.5 kDa, p*I* 6.5) and PG 2 (38 kDa, p*I* 5.4) exhibited quite similar properties, they were found to be both endo-acting enzymes. Both PGs cleaved penta- and trigalacturonic acid while tetragalacturonic acid was only cleaved when trigalacturonic acid was present. The latter substrate was hydrolysed much faster by PG 2. Both enzymes were active on pectins with different degrees of esterification, they were sensitive towards Ca-cations and not glycosylated. The kinetic properties were measured by viscosimetry with polygalacturonic acid as a substrate. NMR experiments on a model substrate revealed an inverting mechanism of carbohydrate hydrolysis for both enzymes.

Keywords: Polygalacturonase; FFE; Purification; Pectinase; Plant pathogen fungus

1. Introduction

Polygalacturonases belong to the complex of pectin degrading enzymes and within those to the depolymerising group acting hydrolytically. Their substrates are pectic substances, which have a common frame-polymer composed from α-1,4-linked galacturonic acid units, more or less esterified [1]. They occur as a structural material in the primary wall and in the middle lamellae of higher plants, showing a high diversity depending on the natural source [2]. PGs are found to be produced by a wide variety of organisms, like bacteria, yeasts, fungi, insects and even plants. The physiological functions of PGs are still in investigation, but the involvement of fungal PGs in the infection processes of plants seem to be evident, one common aspect is often the plant pathogenity of organisms, whereby PGs seem to have an important role within the infection process [3]. Industrially these enzymes are interesting for a growing number of different applications, fruit clarification as the classical application, a new field came up, when the potential of pectic substances as dietary fibres was recognised, the preparation of oligosaccharides from pectic polymers and the latest application in the field of cotton processing, PGs were successfully applied in so-called bioscouring sequences [4]. As a suitable organism for industrial production of PG the fungus *A. niger* is used, not only because of the extraordinary amounts of enzyme produced, but also because concomitant synthesis of several substances is possible [5]. Another well investigated fungus known to produce PG is the plant pathogen basidiomycete *Sclerotium rolfsii*. The strain CBS 350.80 was found to produce extraordinary high amounts of these enzymes, anyway one of the highest reported activity values and therefore explored in detail.

Most polygalacturonases have been purified from the fermentation broth by means of chromatographic methods, especially ionexchange and affinity chromatography were applied, as was reviewed by Ref. [6]. The technique of the so-called free flow electrophoresis (FFE) has been developed for continuous separation of molecular and particular substances, e.g. whole cells, organelles, polymers. Particles are processed in a free fluid system, where no matrices forming networks influence their electrophoretic mobility. Successful separation of proteins by

* Corresponding author.
E-mail address: guebitz@tugraz.at (G.M. Guebitz).

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free flow zone electrophoresis were promising [7,8]. Resolution could be appreciably increased by the introduction of other electrophoretic modes, isoelectric focusing (IEF) and isotachophoresis (ITP). Even so, FFE is until now not a commonly used method for enzyme purification.

Enzymes hydrolysing glycosidic linkages of oligo-, polysaccharides and glycosides utilize two different reaction mechanisms leading to a different configuration of the newly formed reducing end [9]. The hydrolysis can be catalyzed in one chemical step – single displacement mechanism – generating reducing-end products with a configuration of the anomeric carbon reversed to that of the cleaved glycosidic linkage. Such enzymes are called inverting. Another group of hydrolases utilize two chemical steps – double displacement mechanism – to perform the cleavage of the glycosidic linkage. Such enzymes are called retaining. In contrast to cellulases and hemicellulases, there is much less information available about the hydrolysis mechanism of pectinases. In this study, we compare the substrate specificities of two new polygalacturonases from *S. rolfsii*.

2. Materials and methods

2.1. Organism and culture conditions

Sclerotium rolfsii (CBS 350.80) was obtained from the University of Agricultural Sciences, Vienna, Austria. The culture was maintained on potato-dextrose-agar (PDA) plates grown at 30 °C, stored at 4 °C and subcultured monthly. For PG production *S. rolfsii* was grown in 1000 ml Erlenmeyer flasks at 30 ± 1 °C on an orbital shaker at 150 rpm using 300 ml culture medium containing the following ingredients: 42.6 g cellulose, 40 g meat peptone, 2.5 g NH_4NO_3, 1.5 g $MgSO_4$, 1.0 g KH_2PO_4, 0.5 g KCl, and 300 μl trace element solution in 1 l distilled water [10]. The initial pH was adjusted to 5 and the growth medium was sterilized at 121 °C for 15 min prior to inoculation with 1 cm^2 discs of fungus growing on agar plates. In order to induce polygalacturonase activity pectin (1 g l^{-1}) was added to the fermentation broth after being sterilized by UV radiation over night to prevent hydrolysis at the point of inoculation. The fermentation continued for a total of 10 days after which the culture supernatant was harvested by centrifugation at 10,000 × *g* for 10 min.

2.2. Enzyme and protein assay

PG activity was measured by determination of reducing sugars released as a result of hydrolysis of sodium polypectate by a dinitrosalicylic acid (DNS) reagent [11]. The reaction volume was adapted to the volumetric capacity of an Eppendorf vessel. The mixture containing 450 μl 0.25% PGA solution in buffer pH 5 and 50 μl of appropriately diluted enzyme solution was incubated at 50 °C for 5 min and the reaction stopped by addition of 750 μl of DNS. After boiling for 5 min, subsequent cooling and centrifugation the absorbance was read at 540 nm. The reducing sugars formed were quantified using D-galacturonic acid as a standard. Activity on other substrates was measured similarly. One unit (U) was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute.

Protein concentration was routinely determined using the Bradford Reagent from Bio-Rad [12] according to the manufacturer's instructions (Bio-Rad) with bovine serum albumin as standard. Dependent on the concentration range the micro or microplate assay has been applied. All chromatographic runs were monitored for protein by absorbance at 280 nm.

2.3. Enzyme purification

2.3.1. Precipitation

The supernatant of the *S. rolfsii* fermentation was subjected to fractionation by ammonium sulphate precipitation at 50% saturation to remove some proteins, followed by 95% saturation in a second step to gain most of the PG activity. The pellet was resuspended in 50 mM Na-acetate buffer pH 4 and dialysed against distilled water.

2.3.2. Free flow electrophoresis

FFE separations were performed with the ProTeam FFE (Tecan GmbH, Austria) using the isoelectric focussing mode. Hydroxypropylmethylcellulose (HPMC from Sigma, Germany) provided the laminar flow within the chamber. Following media were applied:

Anodic stabilisation medium: 25% (w/w) glycerol, HPMC, 100 mM H_2SO_4.
Separation medium 1: 25% (w/w) glycerol, HPMC, 14.3% Prolyte™ 1, pH ca. 4.
Separation medium 2: 25% (w/w) glycerol, HPMC, 20.0% Prolyte™ 2, pH ca. 7.
Separation medium 3: 25% (w/w) glycerol, HPMC, 14.3% Prolyte™ 3, pH ca. 9.7.
Cathodic stabilisation medium: 25% (w/w) glycerol, HPMC, 100 mM NaOH.
Counterflow medium 25% (w/w) glycerol, HPMC.
Anodic circuit electrolyte (sulfuric acid standard solution 1 mol/l, Riedel-de Häen), cathodic circuit electrolyte (sodium hydroxide ≥ 99%, p.a., ROTH).

The concentration of hydroxypropylmethyl cellulose (HPMC) was dependent on the protocol applied—0.2% (w/w) for the high HPMC protocols, and 0.05% (w/w) for the low HPMC protocol. The sample from precipitation was diluted 1:2 with equal parts of HPMC and glycerol (50% sample + 25% solution of 0.8% HPMC + 25% glycerol) to reach appropriate viscosity and density. Furthermore, 5 μl of a 1% solution of the red acidic dye 2-(*p*-sulphophenylazo)-1,8-dihydroxy-3,6-naphtalenedisulfonic acid trisodium salt was added to 1 ml of sample to ease the optical control of the migration within the separation chamber.

Before every trial the whole system has been prerunned for about 30 min, to equilibrate conditions, like current, temperature and media flow and to prefocus the ampholytes. The quality of the laminar flow across the chamber was checked by a so-called stripe test, whereby coloured p*I* markers were loaded to the chamber and collected in a microplate.

The chamber temperature was kept at a constant temperature of 10 °C by a cooling unit during the run. PGs were localized by activity measurements and most interesting fractions analysed by SDS-PAGE.

2.3.3. Gelfiltration

Samples from low HPMC protocols were directly applied to a gelfiltration column. The concentrated sample (200 μl) was loaded onto a Superdex 200 HR 30/10 (Amersham Pharmacia Biotech) column and proteins eluted by flushing with 50 mM citrate buffer pH 6 containing 0.1 M NaCl. The active fractions were analysed by SDS-PAGE.

2.3.4. Control of pH gradient and pI determination

The pH values of the individual FFE microtiter plate fractions were measured manually with a pH microelectrode (Schott, N5900 A). The p*I* values were calculated by taking the pH according to the active fractions.

2.4. Electrophoresis and staining

SDS gel electrophoresis was performed according to the method of Ref. [13], using 10% gels and Coomassie Blue for protein staining. To detect PG activity directly on the gel an adapted procedure described for cellulases has been applied [14]. SDS was omitted and a 0.3% (w/v) PGA solution incorporated into the separating gel (resulting a final concentration of 0.1%). After electrophoresis and incubation on a sponge, soaked with buffer for 2 h, the gel was stained with 0.02% Congo red solution for 15 min, washed with 1 M NaCl solution for another 15 min and treated with acetic acid solution to increase the contrast.

2.5. Determination of temperature–pH optima and stabilities

The pH–temperature profile for the activity of the PGs was compiled by applying the standard enzyme assay at selected temperatures ranging from 30 to 80 °C and at various pH values between pH 2.5 and 7.9 using substrate solutions

in constant ionic strength citrate–phosphate buffer. In each case the substrate was preincubated at the required temperature.

To evaluate the thermal stability aliquot amounts of desalted, concentrated enzyme samples were diluted with 50 mM citrate buffer of various pH values (pH 3.5, 5, 6.5 and 8) and incubated for fixed time periods at 30, 50 and 70 °C. At time intervals samples were withdrawn, cooled on ice before assaying to determine the residual enzyme activity, using the normal assay procedure.

2.6. Effect of cations and surfactants

Out of a big number of potential inhibitors several have been chosen: Fe(3), Hg(2), Ca, Mg (as chloride salts each), K_2CrO_4, SDS, EDTA, and the surfactants Triton X-100, Cotemoll.

An appropriate amount of purified enzyme solution has been incubated with different concentrations of inhibitors (by dilutions of a 100 mM stock solution) for 10 min at room temperature and activity measured. The starting conditions have been checked out within preexperiments, the concentration range varied from 0.05 to 50 mM, dependent on the inhibitor, the surfactants have been applied in a concentration of 0.05%.

2.7. Degree of glycosylation

Possible glycosylation residues were removed enzymatically by treating pure enzyme samples with a glycosidase and an amidase (both New England Biolabs Inc.).

Deglycosylation with Endoglycosidase H (EndoH): 2 μl of 10× denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) were added to 18 μl of protein sample and heated to 100 °C for 10 min, after cooling down the mixture was incubated in G5 Buffer (2.25 μl of 10× buffer, 50 mM Na-citrate) and 2.25 μl of EndoH at 37 °C for 24 h.

Deglycosylation with peptide:*N*-glycosidase F (PNGase F): 2 μl of 10× glycoprotein denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) were added to 18 μl of protein sample and heated to 100 °C for 10 min, after cooling down the mixture was incubated in G7 reaction buffer (2.5 μl of 10× buffer, 50 mM Na-phosphate), supplemented with 2.5 μl 10% NP-40, and 2.5 μl of PNGaseF at 37 °C for 24 h.

The deglycosylation was controlled by applying treated and untreated samples to a SDS-PAGE.

2.8. Change in specific viscosity

Enzyme action on PGA was followed by measuring the increase in reducing groups and reduction in viscosity. The viscosity was determined at a fixed temperature of 30 °C with an AMVn automated mircoviscosimeter (Anton Paar® GmbH, Graz, Austria). For the calculation, it was necessary to determine the density, performed with the DMA 38 density meter (Anton Paar® GmbH, Graz, Austria) by filling the sample loop with the sample liquid and after a certain equilibration time reading the density. A 975–997.5 μl of a 0.5% (w/v) polygalacturonic acid in 50 mM acetate buffer (pH 5) was preincubated at 30 °C and mixed with 2.5–25 μl of the purified enzymes. The mixture was carefully (to prevent air bubbles) filled into the capillary with 1.6 mm diameter. The capillary was put into the AMVn and the decrease of viscosity was monitored at an angle of 70° till no substantial change was observed. The released reducing sugars of similar reaction mixtures were measured with the DNS method.

2.9. Determination of kinetic parameters

Kinetic properties of the PGs were determined by the measurement of viscosity changes of homogalacturonan solutions in 50 mM acetate buffer pH 5 and 30 °C, incubated with a certain amount of enzyme similar to the above described measurements. Tested substrate concentrations were in the range of 0.5–10 g/l. Michaelis–Menten parameters were calculated from Eadie–Hofsted plots V versus V/[S] plots and Hanes–Woolf plots [S]/V versus [S], where [S] is the substrate concentration and V is the initial rate of hydrolysis. By combination of the two methods, mean values were calculated.

2.10. Release of oligosaccharides

Oligogalacturonic acids of degree of polymerisation 2–5 were prepared by enzymatic hydrolysis of citrus pectin followed by chromatographic purification of the fragments. The reaction of PGs with oligogalacturonic acid was performed in Eppendorfs, in a minimized scale, a total volume of 20 μl, containing 20 mM of oligomer in 10 mM acetate buffer pH 5 and a standardized amount of enzyme, which was evaluated in preexperiments. The reaction temperature was set to 30 °C and the reaction controlled by TLC. Samples were withdrawn out of the reaction mixture in certain time intervals and two times 0.5 μl spotted on a TLC plate (20 cm × 20 cm, aluminium sheets, silica gel 60 from Merck), dried with a hair-drier after every spot to stop the enzyme reaction and put in a chamber saturated with the running solvent 1-butanol–formic acid–water = 2:3:1 (v/v), after the run was complete, the plate was taken out, dried and the oligosaccharide spots were developed with orcinol solution (containing 1 g orcine monohydrate from Fluka in 5 ml H_2SO_4 conc. and 95 ml ethanol) by pouring a homogenous layer over the plate, drying the same and heating up to 100 °C in a dry chamber, where the spots became dark coloured.

2.11. Substrate specificities and side activities

Polygalacturonases were tested for their specificities on following substrates: apple pectin (70–75%) and citrus pectin (63–66%), both Fluka and for their side activities on xylan from birchwood (Roth), mannan from *S. cerevisiae* (Sigma) and CMC (Merck). The experiments were carried out in principle as described before.

The reaction mixtures contained 0.5% of the different substrates in 10 mM acetate buffer pH 5 and always the same amount of enzyme. The reaction temperature was set to 30 °C. Evaluation was done by reducing sugar determination and TLC.

2.12. Elucidation of the reaction mechanism

2.12.1. Sample preparation

The purified enzyme samples of polygalacturonases 1 and 2 were concentrated (Vivaspin 6), simultaneously desalted by washing with D_2O (Sigma) several times and two times lyophilized for being redissolved in D_2O immediately before use in ^1H NMR experiments.

2.12.2. Substrate preparation

PentaGalU-ol was prepared by $NaBH_4$ reduction of the corresponding aldouronic acid and lyophilized three times from D_2O.

2.12.3. Reaction mixtures for ^1H NMR spectroscopy

A 20 mM of pentaGalU-ol was dissolved in 0.05 M deuterized acetate buffer pH 5 and 0.1 ml of a suitable concentrated enzyme solution to 0.4 ml of this substrate added. The amount of enzyme was determined by TLC experiments as described before, and should guarantee the hydrolysis rate to be much higher than mutarotation of the newly formed reducing end. ^1H NMR spectra of the reaction mixture were recorded versus time on a varian unity inova 500 spectrometer at 30 °C. The assignment of important resonances was based on published data [15].

3. Results and discussion

Sclerotium rolfsii was found to produce high amounts of polygalacturonase activity. The substrate specificities and properties of the enzymes responsible for this activity are compared in this study.

3.1. Production of polygalacturonases by S. rolfsii

The cellulose containing basal medium used during these experiments was selected because it assured excellent growth of

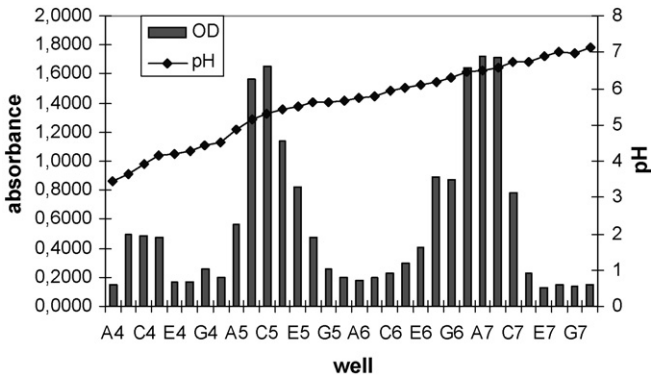

Fig. 1. Analysis of a FFE run, activity distribution of PGs and pH gradient (*X*-axis displays well number according to the common labelling of a microtiter plate).

the fungus, and because cellulose was reported to provide PG production [16]. As a highest value a considerable PG activity of 300 U/ml was measured after 10 days in the basal medium. The amount of PG could be increased by adding pectin, but the extent of 25% was more moderate than one could expect. However, the values for PG activities obtained have been one of the highest reported in literature. The potential of several inducers have been assessed and reported elsewhere [17].

3.2. Native PAGE

In order to know number, molecular weight and p*I* of different PGs, native PAGE is a very helpful tool. The most commonly applied overlay method reported in literature [18] did not work properly, it resulted as its best in large, diffuse spots, but an allocation to certain bands was not possible. The contact area is assumed to be the crucial point of the overlay technique—to allow enzymes diffusing from the gel into the overlay to react with the substrate. A better result was obtained with an adapted method according to Ref. [14], although not described for PGs, as shown in Fig. 1, a slightly visible double band appeared in the middle of the gel.

3.3. Purification of two PGs from S. rolfsii

3.3.1. Precipitation

This step was performed as a purification step to remove unwanted substances and proteins, as well as a concentration step by resuspending the pellet in a less amount of buffer. The best purification effect could be achieved by fractionated precipitation between 50 and 95% saturation of ammonium sulphate. The efficiency of the precipitation step was rather high, considering the reduction of the total protein amount by 42% and the yield of recovered PG of more than 97%.

3.3.2. Free flow electrophoresis (FFE)

Several trials have been run in order to find the best adjustment of the system parameters to optimize focusing, medium flow-rate, sample flow and chamber gap (0.4 mm).

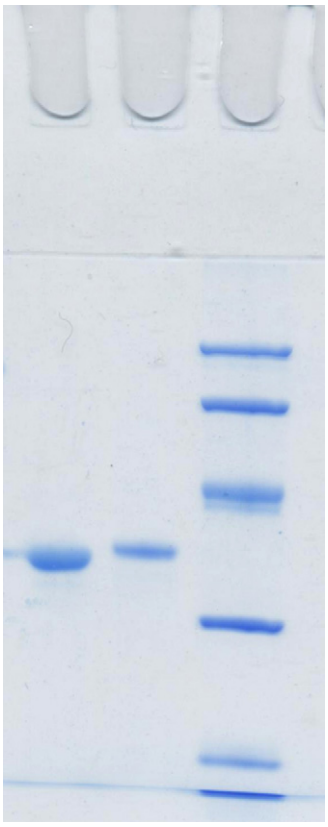

Fig. 2. SDS-PAGE of purified enzymes from FFE; samples applied from left to right: PG2, PG 1 and molecular weight standards (97.4, 66.2, 45, 31, 21.5, 14.4 kDa).

A high HPMC protocol was run to set the conditions and then changed to a low protocol to collect the samples. PG activity measurements revealed three activity peaks at different pH values (Fig. 2). It was conspicuously, that the activity measured for the first peak (well B4, C4 and D4) was much lower compared to both others. SDS (data not shown) analysis revealed a number of proteins being present in these fractions, whereas excellent separation was achieved for fractions B5, C5 and H6, A7 and B7, although there were some other protein bands present. Subsequently the two peaks with the higher activity were selected for further upgrading. For complete purification and for removal of the matrix substances, particularly the HPMC, fractions of the two main peaks were subjected to a gelfiltration step. As displayed in Fig. 3, clear defined bands were obtained indicating pure enzyme fractions.

To calculate the activity retrieval, pumps were calibrated, the sample focused for exactly 10 min and collected in a microplate. Out of 227 IU applied, 219 IU were found in 22 active fractions, 11.4% in peak 1, 27.1% in peak 2 and 58.0% in peak 3—that is a recovery of 96.5%.

3.3.3. Determination of MW and pI

The PGs from FFE have been compared by SDS page analysis and calibration with molecular standards yielded molecular weights as listed in Table 1. The p*I* was averaged out of numerous trials from FFE. The enzyme with the higher p*I* was named PG 1, the one with lower p*I* PG 2.

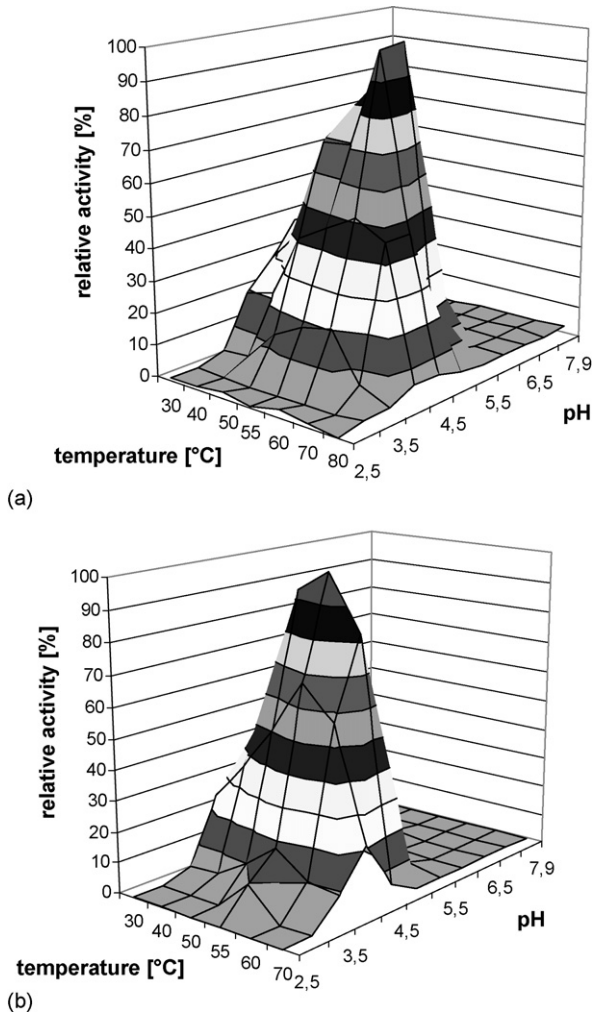

Fig. 3. Activity profiles at different pH and temperature values of (a) PG 1 and (b) PG 2.

The advantages of the FFE method are obvious, continuous separation with high resolution. Detection of number of enzymes with differences in their p*I* and estimation of p*I*, thus it is not only a preparative, but also an analytical method. Out of the FFE experiments informations were obtained, which were of big importance for establishing a successful purification strategy. Reviewing the literature, two reports about purification of PGs from *S. rolfsii* were found. An acid-stable endo-PG with an pH optimum of 2.5 was purified by precipitation and five chromatographic steps Sephadex G-100, DEAE cellulose, Sephadex G-200, SE-Sephadex C-50 and Sephadex G-100 239-fold in a yield of 3.8% [17] and two different polygalacturonases were separated by ammonium sulphate precipitation 80–95% and two chromatographic steps, DEAE-cellulose pH 8 and Sephadex G-75, pH 4.5, revealing a smaller enzyme with an MW of 28–31 and a larger enzyme with 46–48 kDa [19]. These described enzymes differ from the enzymes purified in this work. This is not surprising, since the authors had studied different strains of *S. rolfsii*. Previously, surprisingly high variations in terms of number and amount of hemicellulolytic enzymes, produced by different strains of *S. rolfsii* had been reported [21].

Table 1
Molecular weight and isoelectric point of purified polygalacturonases from *S. rolfsii*

	MW (kDa)	p*I*
PG 1	39.5	6.5 ± 0.2
PG 2	38	5.3 ± 0.1

4. Biochemical characterisation

4.1.1. Activity optima and enzyme stabilities

PG 1 was optimally active at pH 5 and 60 °C while PG 2 had its activity optimum at pH 4.5 and 55 °C. Both enzymes exhibited well defined activity peaks at different pH and temperature values (Fig. 4). PG 1 was quite stable at 50 °C and pH 5 but exhibited more sensitivity against pH values below and above 5; at 70 °C the stabilities were drastically reduced. PG 2 turned out to be a really sensitive enzyme, its half-life time at 50 °C was in the range of minutes and at 70 °C the enzyme was inactivated within 1 min (Table 2).

4.1.2. Effect of Inhibitors

Potential inhibitors of PGs have been tested up to concentrations of 50 mM. Fe(II) could not be examined, since it was immediately oxidised by the DNS reagent giving a positive response even without enzyme and was therefore replaced by Fe(III). I_{50} values are shown in Table 4. PG 1 and PG 2 were

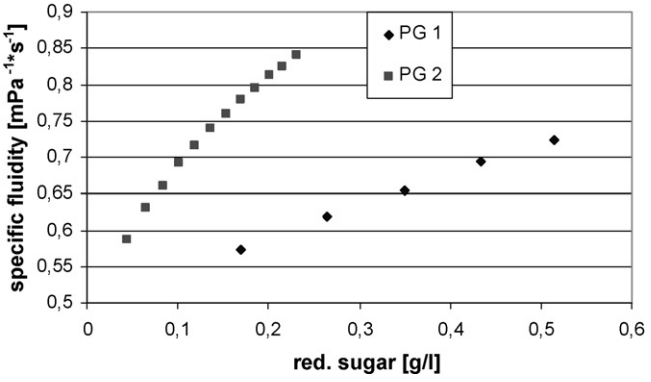

Fig. 4. Relationship between the specific fluidity of polygalacturonic acid and the released reducing sugars during hydrolysis by the purified polygalacturonases of *S. rolfsii*.

Table 2
Half-life times of two polygalacturonases from *S. rolfsii* at different conditions

pH	Temperature (°C)	PG 1	PG 2
3.5	50	160 min	45 min
	70	30 min	<1 min
5	50	48 h	40 min
	70	50 min	<1 min
6.5	50	80 min	5 min
	70	20 min	<1 min

strongly inhibited by Ca(II), but less by Mg(II). Hg(II), Fe(III) and SDS were found to be stronger inhibitors for PG 1 than for PG 2, for Cr(VI) the opposite was true. EDTA and surfactants did not influence enzyme activity. Comparison of these results with literature values is problematic, since different conditions were applied for inhibition experiments. Multiple forms of polygalacturonases from *A. carbonarius* were insensitive towards a number of metal ions including Ca, Mg and Fe, except Hg(II), incubated in 9.5 mM chloride salt for 15 min [22]. A purified PG from *S. sclerotiorum* was 75% inhibited by Ca(II) at 2 mM, but not sensitive to other divalent cations, including Hg(II) and EDTA, respectively [23].

4.1.3. Extent of glycosylation

The extent of glycosylation was determined enzymatically, whereby two different enzymes were applied. Endoglycosidase H is a glycosidase, which cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from *N*-linked glycoproteins and PNGase F is an amidase, which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins.

With both enzymes the same result was obtained. Neither PG 1 nor PG 2 seemed to be glycosylated or could not be revealed by SDS analysis (pictures not shown).

In accordance a PG of *Armillaria sp*. was reported to be not glycosylated as fungal PGs in general were supposed to be [24]. Low degree of glycosylation was found for a PG from *A. alternata* [25] and *M. flavus* [26], where mass differences in the range of a single hexose group and *N*-acetylhexosamine, respectively, were detected. Multiple forms of polygalacturonases from *Aspergillus* sp. have glycoprotein character, whereby deglycosylation lead to complete inactivation of tested enzymes [27]. Similarly, several PGs from *S. sclerotiorum* were found to be glycosylated [28] and differential glycosylation could be responsible for multiplicity of enzyme systems of the same organism [23].

4.2. Substrate specificities and mechanism of hydrolysis

4.2.1. Hydrolysis of oligosaccharides

The two polygalacturonases were incubated with di- to pentamer of α-D-galacturonic acid and the reaction was prosecuted by TLC. None of the PGs hydrolysed digalacturonic acid. Both enzymes cleaved the trimer yielding equal amounts of the di- and monosaccharide. Interestingly, although the enzymes were dosed based on the same activity on polygalacturonic acid (30 IU/ml), complete hydrolysis of the trimer was observed after 6 h in the case of PG 1 while PG 2 acted much faster completely hydrolysing the trimer after 1 h.

None of the enzymes hydrolysed tetragalacturonic acid although this substance seemed to be degraded in the presence of other oligomers. Reaction of PG 1 with the pentamer yielded in several smaller oligosaccharides including tetragalacturonic acid. After 15 min equal amounts of penta- and tetramer were detected and some amounts of tri-, di- and monosaccharide, after 1 h the pentamer was completely hydrolysed and equal amounts of tetra- and dimer came up. After 2 h the tetramer disappeared yielding some trimer and monomer but primarily digalacturonic acid, the trimer was further hydrolysed as described before. PG 2 showed again the same pattern with a higher reaction rate compared to PG 1 (as described for the trimer). The fact that the tetramer was further hydrolysed in the course of pentagalacturonic acid degradation could be an indication of certain steric restrictions within the active site of the enzymes, connoting a number of sub-sites has to be filled with galacturonic acid units in order to provide cleavage of oligomers. This result was confirmed when the enzymes were incubated with equal amounts of tetragalacturonic acid and trigalacturonic acid. Interestingly, in the presence of trigalacturonic acid both enzymes were able to hydrolyse tetragalacturonic acid.

In general PGs exhibit lower reaction rates the shorter the oligosaccharides are. A PG from *Mucor flavus* rapidly hydrolysed oligomers DP 5–7 to mono-, di-, tri- and tetramer, but very slowly the oligomers DP 2–4, which accumulated in all reaction hydrolysates [26]. The PG from *F. moniliforme* hydrolysed several oligomers from DP 3 to 7 while the dimer was not attacked [29].

Table 3
Relative activity in % of two PGs from *S. rolfsii* on different pectins normalized to activity on PGA

	PG 1	PG 2
Apple pectin	9	19
Citrus pectin	13	17
Polygalacturonic acid	100	100

4.2.2. Hydrolysis of different polysaccharides

Apple pectin with a degree of esterification (DE) of 70–75% and citrus pectin with a DE of 63–66% were compared with PGA (all of them in concentrations of 0.5%) (Table 3). Compared to PGA, polymethoxygalacturonic acids like apple and citrus pectin were worse substrates for both enzymes with a more pronounced effect for PG 2. Sakai et al. [1] doubted the existence of a so-called polymethylgalacturonase (PMG), although there were numerous publications about such enzymes produced by *A. niger* [30,31]. Well, in 1997 the same authors reported the expression of a gene encoding for an enzyme in *T. penicilla-*

Table 4
Calculated I_{50} values in mM (values in brackets are relative activities at 50 mmol of corresponding substance)

	PG 1	PG 2
Ca(II)	7	15
Mg(II)	>50 (90%)	>50 (55%)
CrO_4	32	18
Fe(III)	0.75	16
Hg(II)	0.07	0.9
SDS	0.5	12
EDTA	>50 (90%)	>50 (83%)
Triton X-100	(87%)	(91%)
Cotemoll	(82%)	(90%)

tum, which was only degrading polymethoxygalacturonic acid and not PGA [32]. Although PG 1 obviously cleaved apple and citrus pectin as indicated by the release of reducing sugars, no small oligomers were detected using TLC. In contrast, hydrolysis of PGA resulted in the formation of small oligomers. Unlike PG 1, PG 2 released oligomers from apple and citrus pectin as well as from PGA.

Generally PGs show lower activity on esterified pectin. The PG from *A. carbonarius* exhibited only 5% activity on apple and citrus pectin compared to its action on PGA [22] and 2–5% on 75% esterified pectin for several PGs of *A. niger* N400 [33].

None of the two PGs from *S. rolfsii* exhibited activity towards xylan and carboxymethylcellulose as determined by the release of reducing sugars. In contrast TLC experiments revealed some hydrolysis of xylan and CMC for PG 2, but not for mannan, PG 1 showed in agreement to the above mentioned measurements no reaction products.

4.2.3. Mechanism of hydrolysis

4.2.3.1. Viscosimetry. PG 1 and 2 decreased viscosity of a PGA solution rapidly, whereby PG 2 exhibited a faster reduction of viscosity compared to PG 1. The course of viscosity and corresponding reducing sugars is shown in Fig. 4. Both polygalacturonases from *S. rolfsii* show an endo mode of action. Comparing the slopes of the regression curves, PG 2 decreased the viscosity of a PGA solution about four times faster than PG 1 based on the same number of cleavages performed (reducing ends generated) indicating the higher endo character of PG 2. A rapid decrease in viscosity relative to reducing groups liberation indicates that internal glycosidic bonds of the polymer are split by the enzyme preferentially (endo-enzymes) while a relatively slow change in viscosity indicates hydrolysis more at the end of the chains (exo-enzymes) [34]. To be classified as an endo enzyme, the relative viscosity decrease has to be considerably lower than the relative release of reducing sugars [35]. Reducing sugars concentrations are typically below 10% referred to total sugars at a concomitant viscosity reduction of 50% as a result of endo-acting enzymes.

Using viscosimetry, a V_{max} of 0.0041 mPa and a K_M of 5.77 g/l was determined for PG 1, while PG 2 showed a V_{max} of 0.0129 mPa and a K_M of 11.55 g/l.

4.2.3.2. NMR analysis. Reduced pentagalacturonic acid was used as a substrate for PG 1 and 2 to investigate the stereochemistry during hydrolysis. The configuration of the reducing ends in the products formed in D_2O reaction mixtures was monitored by ^{1}H NMR spectroscopy as described previously for PGs from *Aspergillus* sp. [36].

Since PG 1 was reacting much slower on oligomers than PG 2 the double amount of activity was applied: 500 IU/ml for PG 1 and 250 IU/ml for PG 2. PentaGalU-ol was hydrolysed by both enzymes to triGalUA and diGalU-ol. The first cleavage was followed by hydrolysis of triGalUA to diGalUA and GalUA. This mode of attack of the substrates observed by TLC was in agreement with the ^{1}H NMR spectra of the enzyme substrate mixture recorded at various time intervals after enzyme addition.

Both enzymes lead to the same pattern of hydrolysis products according to NMR spectra (Figs. 5 and 6). The signals of pentaGalU-ol were replaced by signals of triGalUA, whereby the

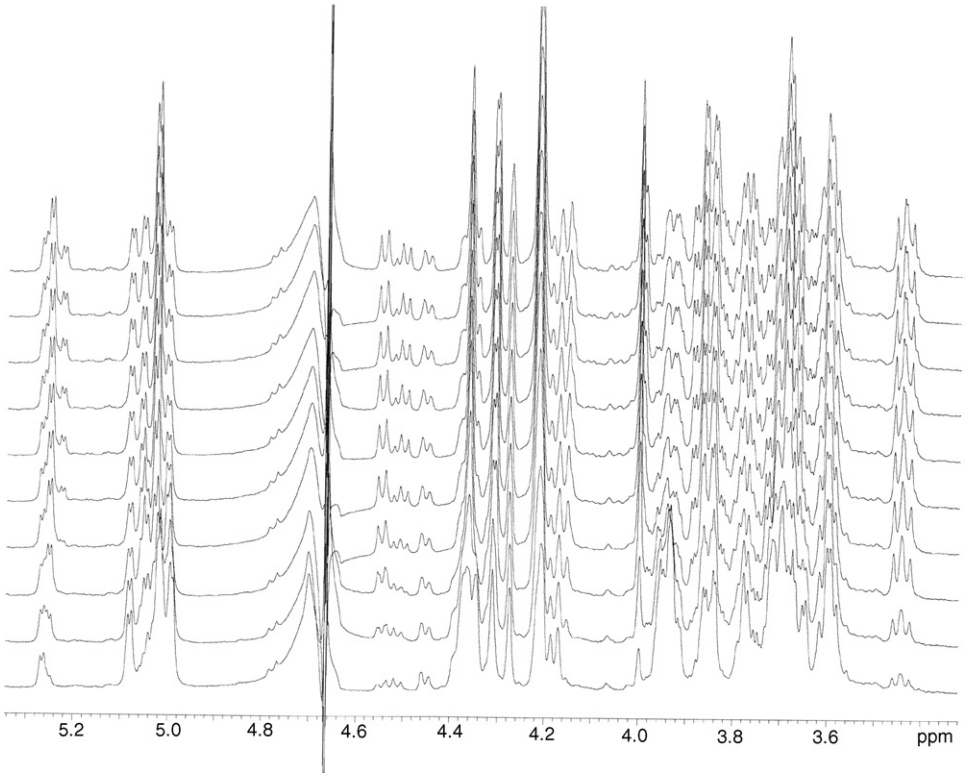

Fig. 5. ^{1}H NMR spectra of pentaGalU-ol hydrolysed by PG 1. Spectra were taken every 30 min (important assignments see text).

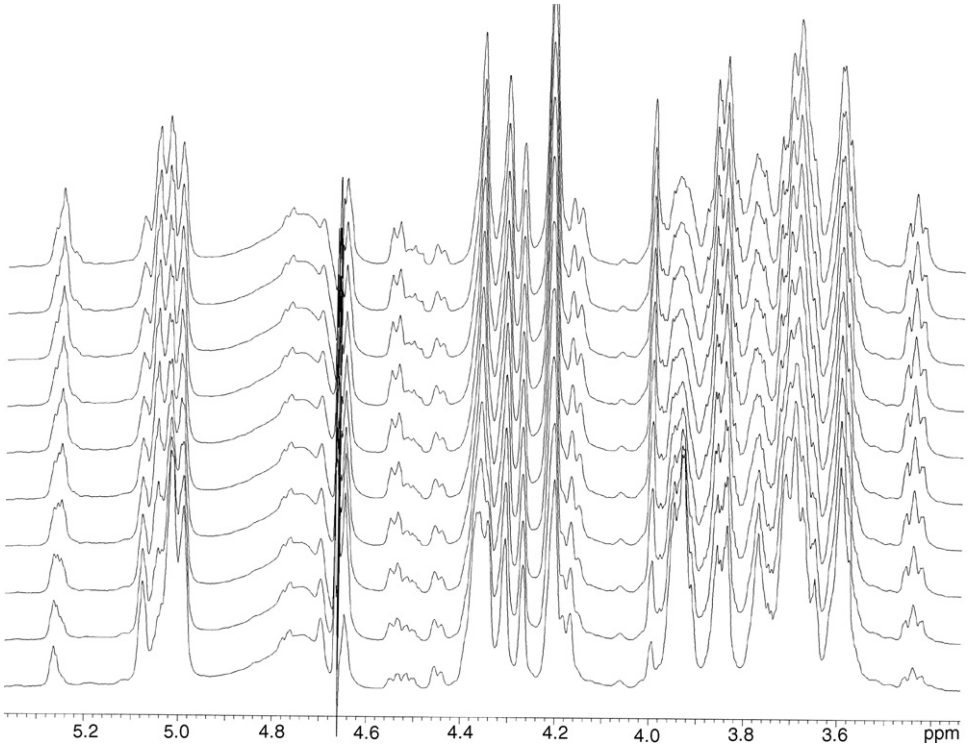

Fig. 6. ^{1}H NMR spectra of pentaGalU-ol hydrolysed by PG 2. Spectra were taken every 5 min (important assignments see text).

β-anomer (H-1β, 4.64 ppm) appeared slightly earlier than the α-compound. Another important easily assignable resonance, which appeared in the spectra immediately after the formation of a new reducing end in β-configuration, is that of the C-2 proton (H-2β multiplet at 3.53 ppm).

There was also some secondary cleavage of the trimer, although the reaction was slow. The pattern obtained was similar to the cleavage of the pentamer, β-GalUA (H-1β, 4.60 ppm) was generated first followed by the α-anomer (H-1α, 5.31 ppm). Although these results indicate an inverting type of hydrolysis mechanism the simultaneously taking place mutarotation was influencing the results.

Summarizing these results, two polygalacturonases from *Sclerotium rolfsii* were purified by the new technology of free flow electrophoresis in isoelectric focusing mode to homogeneity using simply ammoniumsulphate precipitation as preliminary purification step. Both enzymes were endo-acting and inverting the stereochemical configuration of the newly formed reducing end. The described enzymes were applied for bioscouring of cotton [17]. Future experiments will focus on the elucidation of the structure/function relationship of these enzymes to explain the substrate specificities discussed here.

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