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Carbohydrate Research 338 (2003) 515–524
CARBOHYDRATE RESEARCH
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New polygalacturonases from *Trichoderma reesei*: characterization and their specificities to partially methylated and acetylated pectins

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Received 7 June 2002; received in revised form 23 September 2002; accepted 12 October 2002

Abstract

Two extracellular isoenzymes of polygalacturonases PG1 and PG2 were isolated from 3-day-old culture filtrates of *Trichoderma reesei*. The two enzymes were purified to homogeneity by ion-exchange, gel filtration and hydrophobic interaction chromatographies. PG1 and PG2 exhibit similar molecular weights from gel filtration and SDS-PAGE. Their properties, including optimal pH and temperature, thermal stability and *K*m were compared. Characterization of substrate specificity showed that the two enzymes had higher affinity toward PGA (B0100) derived from sugar beet pectin (SBP) than PGA from lime pectin. A series of SBPs with different distribution patterns of methyl and acetyl groups, produced by treatment with either plant pectin methylesterase (P-series) or fungal pectin methylesterase (F-series) or base catalysis (B-series), was used as substrates for PG1 and PG2. Substrates with a low degree of esterification were preferred substrates. The activities of PG1 and PG2 were strongly correlated to the degree of methylation and very little effect from acetylation. The products generated by digestion of selected lime and SBPs were analysed using matrix assisted laser desorption ionisation time of flight (MALDI TOF) MS. A mode of action revealed a random cleavage pattern for PG1 and PG2, confirming that these enzymes are endopolygalacturonases.

Keywords: Sugar beet; Lime; Pectins; Polygalacturonase; MALDI TOF MS

1. Introduction

The polygalacturonases catalyse the hydrolytic cleavage of the O-glycosyl bond of α-D-(1→4)polygalacturonan. The pattern of degradation proceeds in either a random (*endo*-polygalacturonase, EC3.2.1.15) or terminal fashion (*exo*-polygalacturonase, EC 3.2.1.67). Polygalacturonases are produced by higher and lower plants, bacteria and fungi. The role of the enzyme in microbial pathogenesis,[1,2] fruit ripening and softening,[3] abscission,[4] and growth,[5] has been well established. Fungal PGs are also used in industrial applications, such as processing aids for extraction, clarification, and deproteinization of fruit juices, for maceration of fruits and vegetables, and for extraction of vegetable oils.[6,7] Novel fields of application have also recently been envisaged for PGs in the production of oligogalacturonides as functional food components.[6]

PGs from a large number of fungi have been purified and characterised. They show extensive variation in physical and chemical properties.[8–11] However, amino acid compositions of PGs appear quite similar, suggesting that the basic protein structure is maintained among different fungi.[12] Based on their sequences, PGs have been classified in family 28 of the glycosidases.[13] PG-I, -II, -C and -E from *A. niger* were described as inverting enzymes.[14] It could be hypothesised that all PGs follow a similar stereochemistry of the hydrolysis reaction, as the mechanism is conserved among all the members of a given family.[15] To date, one of the best producers of pectin-degrading enzymes is *A. niger*.

Abbreviations: PG, polygalacturonase; PGA, polygalacturonic acid; DP, degree of polymerization; HG, homogalacturonan; PME, pectin methylesterase; DE, degree of esterification.

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0008-6215/03/$ - see front matter
PII: S0008-6215(02)00398-1

Trichoderma reesei has a long history of safe use in industrial-scale enzyme production. Applications of cellulases and xylanases produced by this fungus are found in food, animal feed, pharmaceutical, textile and pulp and paper industries. *T. reesei* is non-pathogenic for man and it has been shown not to produce fungal toxins or antibiotics under conditions used for enzyme production.[16] This is the first report on the purification and characterization of two polygalacturonases from *T. reesei* with high activity toward acetylated and methylated homogalacturonans. Base-treated sugar beet pectin (SBP) (B0915), containing methyl and acetyl groups, was used as an inductive carbon source in culture. We also studied the substrate specificities of the enzymes using a series of SBPs with varying degree of acetyl and methyl esterification. The degradation products were analysed by matrix assisted laser desorption ionisation mass spectrometry (MALDI) TOF MS.

2. Experimental

2.1. Synthesis of model pectins

The partially methyl- and acetylesterified pectins were produced by Danisco (Dr Hans Christian Buchholt, Brabrand, Denmark) and comprised six series, three from SBP and three from lime pectin (E81, a regular commercial pectin with 81% methylesterification). For the first model series, starting materials were deesterified by a fungal (*Aspergillus* sp.) pectin methylesterase (F-series). For the second series, starting materials were treated with a plant pectin methylesterase (P-series). The third series was obtained by alkaline treatment of starting materials (B-series). Finally, a fully deesterified lime pectin, also obtained by saponification, called lime polygalacturonic acid, served as a control. For the SBP series, the first two numbers designated the degree of methylation and the second two numbers the degree of acetylation. For the lime pectin series the number designated only the degree of methylation.

2.2. Organism and growth conditions

The *T. reesei* ATCC 26920 is maintained on potato dextrose agar plates with 0.1% base-treated SBP (B0915) at 5 °C. The extracellular enzyme was produced by fermentation of this fungus in shaking flasks using potato dextrose broth containing 0.1% of B0915. The shaking flasks were incubated for 3 days at 30 °C and 200 r.p.m on a laboratory shaker before the supernatant was recovered by filtration.

2.3. Purification of PG

Purification of *T. reesei* PG was followed by measuring the activity towards polygalacturonic acid. The cell-free broth was concentrated using ultrafiltration (Amicon cut value 10 kDa) and dialysed against 20 mM Tris–HCl buffer pH 7.0. Dialysed material was loaded onto a DEAE–Sepharose CL-6B column (10×1.6 cm i.d) equilibrated with 50 mM Tris–HCl buffer pH 7.0. The enzymes were eluted with a linear gradient from 0.0 to 0.5 M NaCl in the same buffer. Two peaks of active fractions were pooled and concentrated using ultrafiltration and designated PG1 and PG2. The two enzymes were separately loaded onto HiPrep 16/60 Sephacryl S-200 HR column (Amersham BioScience) equilibrated with 20 mM Tris–HCl buffer pH 7.0 containing 0.1 M NaCl. The active fractions of PG1 were dialysed against 50 mM sodium acetate buffer pH 5.0 using ultrafiltration and loaded onto a HiTrap SP-Sepharose FF column (5×1 mL, Amersham Biosience) and the enzyme was eluted with a stepwise gradient from 0.0 to 0.5 M NaCl in the same buffer. The enzyme was eluted very early at 0.05 M NaCl. The active fractions of PG2 after Sephacryl column were dialysed against 50 mM sodium acetate buffer pH 5.0 using ultrafiltration and applied on a HiTrap Phenyl Sepharose FF column (5×1 mL, Amersham BioScience) equilibrated with 1.8 M $(NH_4)_2SO_4$ in sodium acetate buffer pH 5.0. Bound proteins were eluted with a linear decreasing gradient from 1.8 to 0.0 M $(NH_4)_2SO_4$. The fractions containing enzyme activity were collected and dialysed against 50 mM sodium acetate buffer pH 5.0 using ultrafiltration. The last step in the purification of PG1 and PG2 was the application of the concentrated samples to gel filtration (Superdex 75 HR 10/30, Amersham BioScience) equilibrated with 50 mM sodium acetate buffer pH 5.0 containing 0.1 M NaCl using FPLC system. The proteins were eluted at a flow rate of 20 mL/h for all columns. All steps were performed at 4 °C.

2.4. Enzymatic assay

PG activities were calculated from the increase in reducing ends,[17] using galacturonic acid as standard. Reaction mixtures contained 0.9 mL substrate (1 g/L) incubated with 0.1 mL appropriately diluted PG at 40 °C. The hydrolyses were carried out in 50 mM sodium acetate buffer pH 4.5. Reducing end assays were carried out in microtiter plates.[18] One unit of enzyme activity is defined as the amount of enzyme which increases the O.D. 1.0 per min per mL under standard assay conditions.

2.5. Protein determination

Protein content was determined by the dye binding assays.[19] Protein dye reagent was purchased from Bio-Rad Laboratories. Bovine serum albumin was used as the standard.

2.6. Molecular weight determination

The native molecular weight was determined by a Superdex 75 HR 10/30 using a molecular weight calibration kit from Amersham BioScience. The subunit molecular weight was determined by electrophoresis of purified enzyme on 10–20% Tricine gel (Novex) using denatured conditions at pH 8.8 as described by Laemmli.[20] A molecular weight calibration kit (Amersham BioScience) was loaded under the same conditions to allow the determination of the molecular mass. The proteins were stained by silver nitrate.

2.7. Enzyme characterization

Estimates of optimal temperature and pH were made by using a temperature range of 10–90 °C and a pH range of 3.5–5.5. The thermal stability was investigated by measuring the residual activity of the enzymes after 15 min of incubation at different temperatures. The km values were determined from Lineweaver–Burk plots by using substrate concentrations from 0.05 to 0.54 mg/mL.

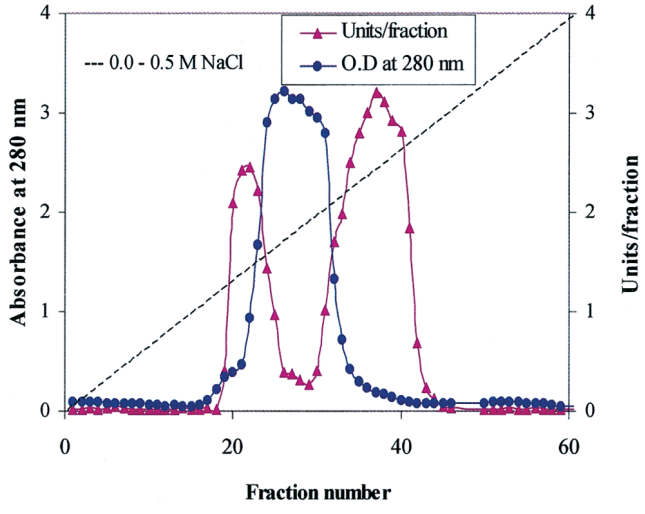

Fig. 1. A typical elution profile for the chromatography of *T. reesei* on DEAE-Sepharose column (10 × 1.6 cm i.d.) previously equilibrated with 50 mM Tris–HCl buffer, pH 7.0 at a flow rate of 20 mL/h and 2 mL fractions.

2.8. Enzymatic digestion

A pectin sample was dissolved in 50 mM sodium acetate buffer pH 4.5 (for PG1) and 4.2 (for PG2 and *A. niger* PGII) at a concentration of 5 mg/mL and incubated with purified enzyme (0.1 U) for a defined period of time at room temperature. The thermal stability was investigated by measuring the residual activity of the enzyme after 15 min of incubation at different temperatures. The reaction was stopped by treating the samples in a boiling water bath for 5 min.

2.9. Mass spectrometer

MALDI time-of-flight (TOF) spectra were acquired on a PerSeptive Biosystems Voyager-DE using delayed ion extraction (delay time 100 ns). To avoid saturation of detector gating (cut-off at 500 Da) was used. The instrument was calibrated externally in negative ion mode using the peptides angiotensin I and ACTH.[21,22]

2.10. Sample preparation for MALDI TOF MS

2,4,6-Trihydroxyacetophenone (THAP, HPLC grade; Aldrich) was dissolved in MeOH to a concentration of 200 mg/mL. Nitrocellulose (Trans-blot transfer medium, 0.45 μm; BioRad) was dissolved in acetone to a concentration of 30 mg/mL and diluted with isopropanol to a final concentration of 15 mg/mL. THAP and nitrocellulose solutions were mixed in the ratio 4:1. A 0.2 μL volume of this matrix solution was placed on the metal target. The solution spread out rapidly, forming a thin layer of homogeneous, very fine crystals. Analyte solutions were desalted using home-made miniaturised columns (2 μL volume) containing about 1.5 mg ammonium loaded cation exchange resin (50W-X8, 200–400 mesh, hydrogen form; Bio-Rad) as described earlier.[23] The analyte solution (1.5 μL) was passed through the column and spotted directly onto the matrix layer.

3. Results

3.1. Production of PG

The production of PG by *T. reesei* was induced by addition of the model SBP (B0915). PG activity reached its maximum at about 3 days of incubation at 30 °C (data not shown).

3.2. Purification of PG

By DEAE–Sepharose column two polygalacturonases PG1 and PG2 were separated (Fig. 1). Further purification of PG1 and PG2 was carried out by two series of

Table 1
Purification scheme for *T. reesei* polygalacturonases

Purification step	Total units	Total protein (mg)	Specific activity (units/mg)	Fold purification	Recovery %
Crude extract	44.5	4.98	8.93	1	100
DEAE–Sepharose					
PG1	10.5	0.42	25	2.79	23.6
PG2	19.1	0.28	68.2	7.6	42.9
PG1					
Sephacryl S200	7.8	0.031	251.6	28.1	17.5
SP-Sepharose	6.2	0.011	563.6	63.1	13.9
Superdex	5.5	0.006	916.6	102.6	12.3
PG2					
Sephacryl S200	12.7	0.067	189.9	21.2	28.5
Phenyl-Sepharose	9.5	0.032	296.8	33.2	21.3
Superdex	8.5	0.012	708.3	79.3	19.1

steps, Sephacryl S-200, SP-Sepharose and Superdex 75 for PG1, and Sephacryl S-200, Phenyl-Sepharose and Superdex 75 for PG2, respectively. The specific activities for the purified PG1 and PG2 was 917 and 708 units/mg protein. These correspond to purification fold of 103 and 79 for PG1 and PG2, respectively. The purification of PG1 and PG2 is summarised in Table 1. The purified PG1 and PG2 were homogenous as judged by SDS-PAGE (Fig. 2), where one protein band was detected on the gel for each enzyme. Molecular weights of the PG1 and PG2, as assessed by gel filtration chromatography, were 66 and 63 kDa. This is in agreement with that observed by SDS-PAGE (66 kDa).

3.3. Characterization of PG1 and PG2

The pH optima of *T. reesei* PG1 and PG2 were 4.5 and 4.2 and their activities dropped rapidly at pHs 3.5 and 5.0, respectively (Fig. 3). The temperature optima of PG1 and PG2 were 40 and 50 °C, respectively (Fig. 4). PG1 was almost inactive at 60 °C, while PG2 exhibited 75% of its activity at that temperature. PG1 and PG2 were stable up to 40 and 60 °C, and lost most of their activities at 60 and 80 °C, respectively (Fig. 5). The Km's of PG1 and PG2 were 0.15 and 0.93 mg/mL, respectively (Fig. 6).

The mode of action PG1 and PG2 were tested with a series of SBPs with different distribution patterns of methyl and acetyl groups. These model pectins were produced by treatment with either plant pectin methyl esterase (p-PME), fungal (f-PME) or base. The activity was calculated relative to lime polygalacturonic acid which was arbitrarily assigned 100% activity (Table 2). The PGA derived from SBP (B0100) was a better substrate for PG1 and PG2 than lime polygalacturonic acid with 60 and 24% increase in their activities, respectively. SBP B0915, with low degree of methylation (9%) compared to a high degree of acetylation (15%), retained a high percentage of residual activities for PG1 (94%) and PG2 (81%). The activities of PG1 and PG2 with SBPs decreased as the degree of methylation increased and did not depend on their acetylation. PG1 and PG2 had, as expected, very low activity toward the highly esterified SBPs (E-series). The substrate specificity of PG1 and PG2 from *T. reesei* was compared with PGII from *A. niger*. In contrast to the *T. reesei* enzymes, PGII had higher affinity toward lime PGA than all the SBPs, from B-series (Table 2). In addition, PG1 and PG2 were also tested with another series of lime pectins and the results were compared with PGII as previously reported by Pages et al.[10] (Table 3). PG1 had higher activity towards the pectins B15, B34, B43, F11, and F31 than that for lime PGA. Its activity was increased in the range of 9–65%. PG2 was only more

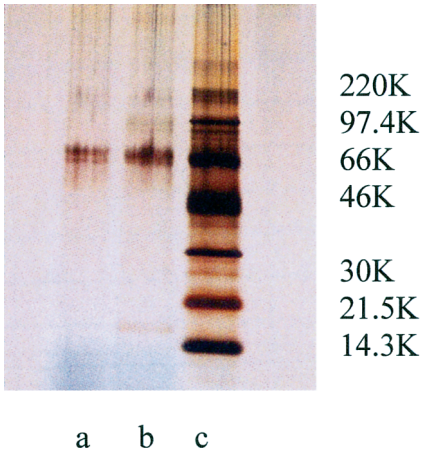

Fig. 2. SDS-PAGE of molecular weight determination of *T. reesei* PG1 and PG2. (a) PG1; (b) PG2; (c) standard proteins: myosin (220 K); phosphorylase b (97.4 K); bovine serum albumin (66 K); ovalbumin (46 K); carbonic anhydrase (30 K); trypsin inhibitor (21.5 K), lysozyme (14.3 K).

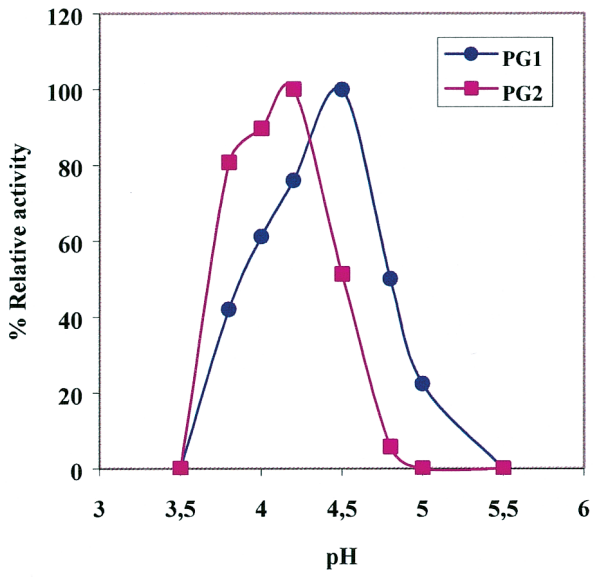

Fig. 3

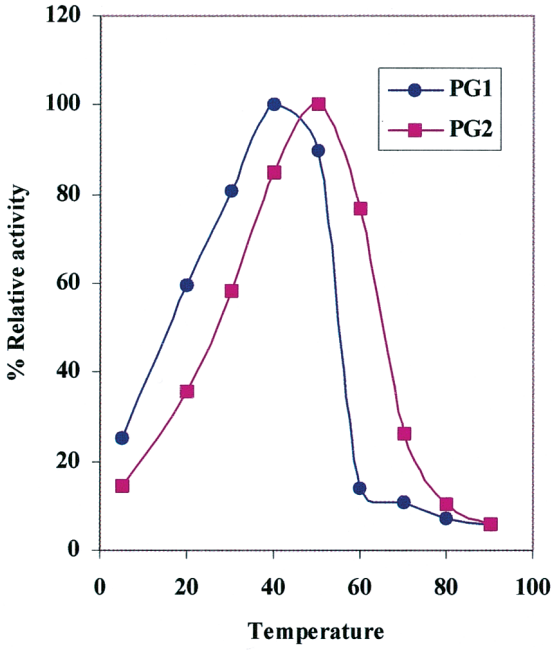

Fig. 4

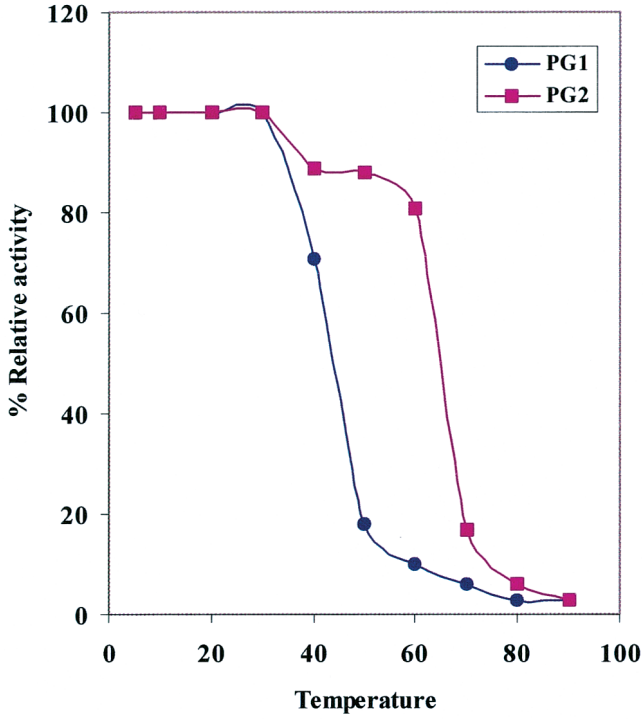

Fig. 5

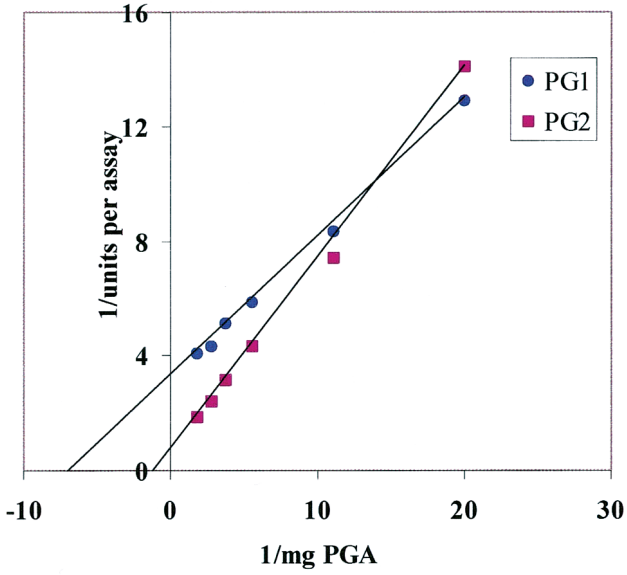

Fig. 6

Fig. 3. pH optima of *T. reesei* PG1 and PG2. Fig. 4. Temperature optima of *T. reesei* PG1 and PG2. The assays were incubated at different temperatures ranging from 5 to 90 °C. Fig. 5. Effect of temperature on stability of *T. reesei* PG1 and PG2. The assays were incubated at various temperatures for 15 min prior to substrate addition, followed by cooling in an ice bath. Activity at zero time was taken as 100% activity. Fig. 6. Lineweaver–Burk plots relating *T. reesei* PG1 and PG2 reaction velocity to PGA concentration.

active to B15. PG1 and PG2 had more affinity toward most lime pectins than PGII. The enzymatic activities also decreased with increased degree of methylation of lime pectins.

The end products of digestion from the different model pectins with PG1, PG2 and *A. niger* PGII were analysed by MALDI TOF MS. Products formed during the very early stages of the reaction were compared

with those present at intermediate and final stages of enzymatic reactions. A range of oligogalacturonides was observed (Table 4). After digestions for 5 mins of the lime pectins B34, F31 and P41, PG1 and PG2 released shorter oligomers with a DP of 3–4 containing both free and esterified products. With the exception of the degradation of P41 by PG1, most oligomers were non-esterified and DP ranged from 3 to 9. For the same time of incubation, the end products of *A. niger* PGII for these three pectins had DP ranged from 3 to 14. Varying degree of methylation was observed for B34 and F31, but only free galacturonic acid oligomers was found with P41. For SBP B0915, few oligomers were produced in the initial phase of the reaction for all three enzymes. With increasing digestion time, different oligomers were produced ($\leq$ DP 14) with high DE for B34 and F31, and low DE for P41. In the case of B0915, the oligomers produced by PG1 had a low degree of methylation and acetylation compared to PG2 and PGII. At the end of the digestion, the tri- and tetramer oligomers were the major products, indicating that most of the long chain oligomers were cleaved to the short chain oligomers. Figs. 7 and 8 show some selected degradation patterns for B34 (lime pectin) and B0915 (SBP) by PG1, PG2 and PGII. After 5 min of

Table 2
Relative activity of *T. reesei* PG1, PG2 and *A. niger* PGII towards different models of SBPs (0.1% substrate/assay)

Pectins	% Relative activity		
	PG1	PG2	PGII
PGA (Lime)	100	100	100
B0100	160	124	90
B0915	94.5	81.4	66
B2516	47.4	50.7	41
B3124	34.3	23.2	24.7
B4626	15.9	11.3	9.5
B5326	11.7	8.0	8.5
F2830	44.7	41	n.d.
F3331	33.9	24.8	n.d.
n.d.			
F4429	19.8	16.4	n.d.
F5129	21.3	11.4	n.d.
P3429	35.6	45.2	n.d.
P4628	28	29.7	n.d.
P5323	18.5	17.4	n.d.
E7327	4.3	6.4	n.d.
E8614	2.1	3.2	n.d.
E9409	1.2	1.0	n.d.
SBP6230	14.8	10.9	5.2

E: highly esterified SBP. SBP: untreated sugar beet pectin. n.d.: not determined.

Table 3
Relative activity of *T. reesei* PG1 and PG2 towards different models of lime pectins (0.1% substrate/assay)

Pectins	% Relative activity		
	PG1	PG2	PGII [a]
PGA	100	100	100
B15	143	121	62
B34	144	101	57
B43	109	95	48
B64	7	18	n.d.
B71	2	8	n.d.
F11	165	99	n.d.
F31	126	76	59
F43	91	59	48
F58	23	19	24
F69	19	19	13
F76	16	8	12
P41	101	90	84
P46	98	78	n.d.
P53	69	40	n.d.
P60	46	38	41
P66	20	34	n.d.
E81	5	6	7

E81: highly esterified lime pectin.
[a] Pages et al.[10]

Table 4
Digestion data of model pectins with 0.1 units of *T. reesei* PG1 and PG2 and *A. niger* PGII at different incubation times analysed by MALDIMS in negative ion mode

Pectin	Time of incubation (h)	PG1 DP	PG2 DP	PGII DP
B0915	4	3–10	3–12	3–13
	24	3–7	3–11	3–12
	48	3–10	3–14	3–12
B34	5 (min)	3–4	3–4	3–14
	4	3–14	3–13	3–14
	24	3–14	3–11	3–12
	48	3–14	3–13	3–14
F31	5 (min)	3–4	3–4	3–10
	4	3–12	3–14	3–14
	24	3–12	3–11	3–10
	48	3–14	3–10	3–13
P41	5 (min)	3–9	3–4	3–12
	4	3–8	3–10	3–6
	24	3–7	3–3	3–6
	48	3–6	3–6	3–6

DP: degree of oligogalacturonoids polymerisation. B0915: SBP. B34, F31, P41: lime pectins.

digestion of B34 with PG1 and PG2, similar and short DP oligomers was produced. Varying methylated oligomers were produced by PG1 and PG2 after 24 h of digestion and reached up to DP 14 and 11, respectively, with high amount of short oligomers up to DP 5. In contrast, PGII produced long chain methylated oligomers ($\leq$ DP 14 and 12) after 5 min and 24 h, respectively. After 4 h of digestion of B0915 by PG1 (Fig. 8), high amount of short oligomers ($\leq$ DP 7) with low degree of methylation and acetylation were observed. In contrast only low amount of long DP oligomers (≤ 10) with high degree of esterification were observed. Homogenous oligomers ($\leq$ DP 7) with very low degree of esterification were produced after 24 h of digestion. The patterns of products produced by digestion of B0915 with PG2 and PGII were similar, where highly DP oligomers up to 12 with varying degree of esterification were detected.

4. Discussion

Methyl esterification is widespread in native (very often high-methoxyl) pectins, e.g., mango (54%), sugar beet (65%), lime (65%), and sunflower (81%). The degree of acetylation however is generally low in native pectins, with some exceptions, e.g., sugar beet and apple pectins (35%).[27] Despite the fact that most naturally occurring pectins contain high degree of esterification, *A. niger* PGs display a low tolerance or preference for partially methylesterified pectins.[10,24,25] It could therefore be of interest to find new sources of enzymes in order to

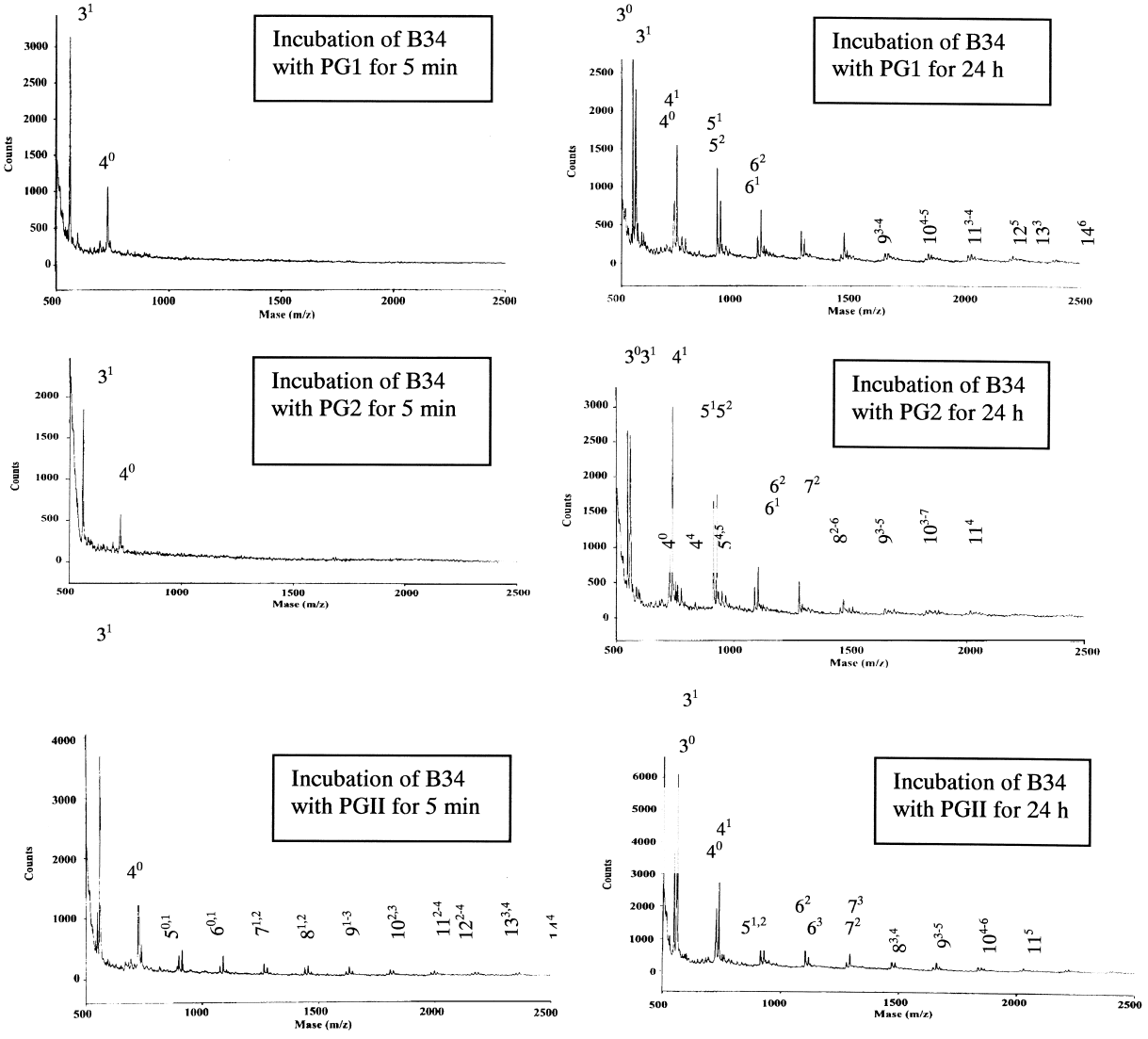

Fig. 7. Digestion of lime pectin B34 with *T. reesei* PG1 and PG2, and *A. niger* PGII at different incubation times analysed by MALDIMS in negative mode. $[M-H]^-$ ions are labelled by giving the DP in normal numbers and the number of methyl ester groups in superscript numbers.

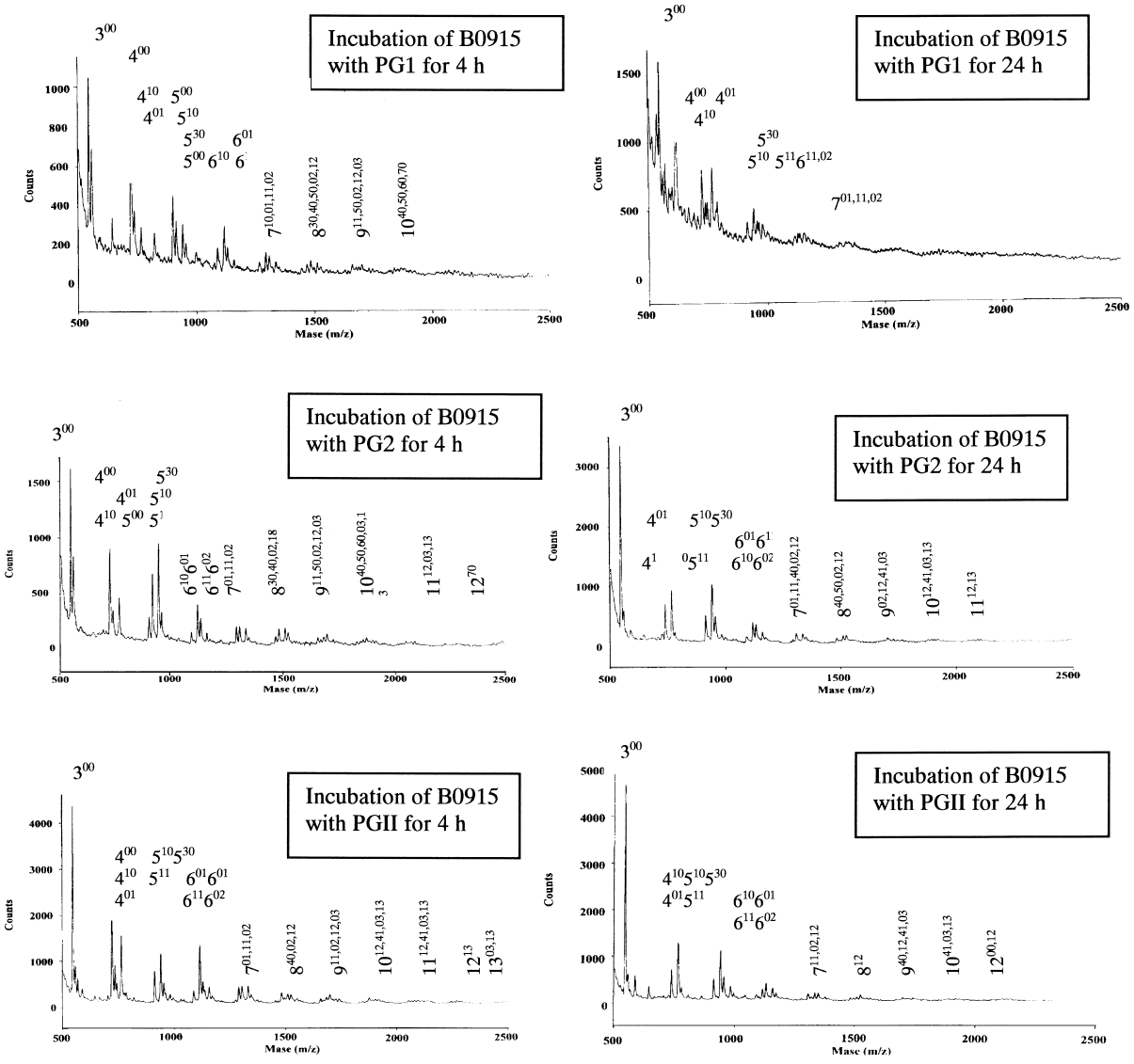

Fig. 8. Digestion of SBP B0915 with *T. reesei* PG1 and PG2, and *A. niger* PGII at different incubation times analysed by MALDIMS in negative mode. $[M-H]^-$ ions are labelled by giving the DP in normal numbers, and the first and second number of methyl and acetyl ester groups in superscript numbers, respectively.

achieve a higher preference for partially methyl and acetyl esterified pectins. In the present study, the extracellular PG was secreted when *T. reesei* was grown in liquid medium containing SBP (B0915) with high degree of acetylation (15%) and low degree of methylation (9%). Usually the organism is grown in culture with polygalacturonan as the carbon sources and the optimal substrate for PG is polygalacturonan.[26] We purified *T. reesei* PG1 and PG2 and used of models of SBP (with methyl- and acetyl esterification) and lime pectin (with methyl esterification) to characterise the enzymes.

In comparison with PGs characterised from other fungi, PG1 and PG2 were monomeric proteins with molecular weight of 66 kDa. This was in agreement with those reported for *Sclerotina sclerotiorum*,[28,29] *Fusarium oxysporum*,[30] and *Colletotrichum lindemuthianum*.[31] The optimal pH and temperature of *T. reesei* PG1 and PG2 were 4.5 and 4.2, and 40 and 50 °C, respectively. All PGs exhibit optimal pH in the acidic region between 3.5 and 5.5,[9,32,33] and optimal temperature between 40 and 55 °C.[8,32] *T. reesei* PG1 appears to have a significantly higher affinity for PGA (Km 0.15 mg/mL) than PG2 (Km 0.93 mg/mL). The Km of the reported polygalacturonases vary from 6.7 mg/mL for *Aspergillus carbonarius* enzyme[34] to 0.12 mg/mL for the *Fusarium moniliforme* enzyme.[33]

The relative reaction rates of *T. reesei* PG1 and PG2 for different models of sugar beet and lime pectins were

measured to clarify their substrate specificity. PG1 and PG2 had higher affinity toward PGA derived from SBP (B0100) than lime PGA. On the contrary, *A. niger* PGII had slightly higher affinity toward lime PGA than sugar beet PGA. PG1 and PG2 had more affinity toward B-series of SBPs than *A. niger* PGII. The results of specificity of SBPs indicated that the decrease in activity was correlated strongly with the increase in degree of methylation, whilst the degree of acetylation had very little effect. When PG1 and PG2 were tested with a series of lime pectins with different degree of methylation, PG1 was highly active on the pectins with degree of esterification up to 43% for B- and F-series, while PG2 is only highly active up to degree of esterification 15% (B15). The same series of lime pectins was tested with *A. niger* PGII, PGI, and PGII mutants E252A and N186E. The enzymes showed higher affinity toward lime PGA than methylated lime pectins.[10] In addition, the distribution of the methylesterification groups also resulted in different relative activities, where PG1 and PG2 had higher affinity toward P-series of lime pectins with high degree of esterification (above 60%) compared to B- and F-series with the same degree of esterification (Table 4). In general, substrates with a low degree of esterification are preferred.[35] As the degree of esterification increases (usually above 20%), Vmax decreases and Km increases.[36] PGs preferentially attack pectate over pectin and their activities decrease as the degree of methylation increases.[37] Barnby et al.[38] found that the activity of *endo*-PG from *K. marixan* with 37.8% esterified pectin is about 95% and with 61% pectin esterification, the activity decrease to 25%. Likewise, in *S. cerevisiae* 1389 and IM1-8b, when 30% esterified pectin was used, the activity decreased by about 30% and when the methylation was 70%, it decreased by approx 60%.[39] On the other hand, the substrate specificity of some fungal PGs was studied using different DP of oligogalacturonic acid, where *Stereum purpureum* PG could hardly hydrolyse with DP lower than 5.[40]

PGs in combination with MALDI-TOF can be used as an efficient tool to determine the fine structure of pectins. Further knowledge about the model pectins, however, is required. The three different series of model pectins have been characterised as follows: (a) for P-series, the action of p-PME is believed to follow a single chain mechanism and results in the consecutive removal of a number of neighbouring methyl ester groups.[41] This gives rise to block-structures of adjacent free galacturonic acid units on the homogalacturonan (HG) backbone; (b) for F-series, de-esterification by f-PME is believed to result from a multiple chain mechanism, which is believed to lead to a random removal of methyl ester groups;[41] (c) for B-series, chemical de-esterification using base catalysis is like f-PME treatment, also believed to produce a random methyl esterification pattern.[42] The MALDI TOF method[23] was applied to analyse the products formed by enzymatic digestion of partially methyl esterified HG. The pattern of oligogalacturonides produced at the beginning of the degradation of B34, F31 and P41 with the three enzymes tested indicated that these enzymes cleaved in the randomly esterified HG for B34 and F31 and nonesterified block-wise HG for P41. With the same time of incubation, only a few oligomers were produced with B0915, indicating strongly that the enzymes had a low tolerance to this substrate containing both methyl and acetyl groups. Generally, most plant cell wall pectins, degraded by pathogenic fungal PGs, have only a varying degree of methyl groups. The digestion of P41 by the three enzymes was characterised by liberation of lower DP oligomers with low DE compared to the B34 and F31. Therefore, PG1, PG2 and PGII had highest affinity toward P41 than B34 and F31. In addition, PG1 had lowest tolerance for B0915 than PG2 and PGII, because PG1 produced low DE oligomers. Of all *A. niger* PGs, PGII has the lowest tolerance for methylesterified substrates.[24,25] Our previous work reported that the increased fragmentation of f-PME treated pectins by PGII can be explained since f-PME function in nature synergistic with PGII to accomplish a very effective breakdown of plant cell walls by the fungal attack. This implies that f-PME produces a pattern of methylesterification that favours *endo*-PG II attack.[42] The pattern of the oligomers produced by degradation of different pectin models confirmed that the P-series had a block-wise pattern of free galacturonic acids on HG, while the B- and F-series had randomly esterified HG. In addition, the mode of action revealed a random cleavage pattern for PG1 and PG2, confirming that these enzymes are indeed endo-polygalacturonases.

Acknowledgements

We want to thank Dr J. Benen for gift of the enzyme *A. niger* PGII and Dr. H.C. Buchholt for the preparation of all of the model pectins. Furthermore, Clive Phipps Walter, Jytte Rasmussen, Hanne Hoberg and Henrik Carlsen should be acknowledged for excellent technical assistances. We thank Cirius (The Danish centre for international cooperation and mobility in education and training) for submitting the research fellowship. Financial support from the European Community ERBIO4CT960685 is gratefully acknowledged.

References

1. Prade, R. A.; Zhan, D.; Ayoubi, P.; Mort, A. J. *Biotechnol. Genet. Eng. Rev.* **1999**, *16*, 361–391.

2. Herron, S. R.; Benen, J. A.; Scvetta, R. D.; Visser, J.; Jurnak, F. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8762–8769.
3. Brummell, D. A.; Harpster, M. H. *Plant Mol. Biol.* **2001**, *47*, 311–340.
4. Riov, J. *Plant Physiol.* **1974**, *53*, 312–316.
5. Pressey, R. In: Fishman, M.L.; Jens, J.J. (Eds.) *Chemistry and Function of Pectins*, American Chemical Society Symposium Series No. 310, Washington, D.C., 1986; pp. 157–174.
6. Lang, C.; Dornenburg, H. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 366–375.
7. Kashyap, D. R.; Vohra, P. K.; Chopra, S.; Tewari, R. *Bioresour. Technol.* **2001**, *77*, 215–227.
8. Keon, J. P. R.; Waksman, G. *Appl. Environ. Microbiol.* **1990**, *56*, 2522–2528.
9. Clausen, C. A.; Green, F. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 750–754.
10. Pages, S.; Heijne, W. H. M.; Kester, H. C. M.; Visser, J.; Benen, J. A. E. *J. Biol. Chem.* **2000**, *275*, 29348–29353.
11. Bonnin, E. M.; Le Goff, A.; Korner, R.; van Alebeek, G. W.; Christensen, T. M.; Voragen, A. G.; Roepstorff, P.; Caprari, C.; Thibault, J. *Biochim. Biophys. Acta* **2001**, *1526*, 301–309.
12. Cervone, F.; De Lorenzo, G.; Salvi, G.; Camardella, L. In *Biology and Molecular Biology of Plant Pathogen Interactions*; Baily, J. A., Ed.; Springer-Verlag KG: Berlin, 1986; pp 385–392.
13. Henrissat, B.; Davies, G. *Curr. Opin. Struct. Biol.* **1997**, *7*, 637–644.
14. Biely, P.; Benen, J. A. E.; Heinrichova, K.; Kester, H. C. M.; Visser, J. *FEBS Lett.* **1996**, *382*, 249–255.
15. Henrissat, B. *Biochem. Soc. Trans.* **1998**, *26*, 153–156.
16. Nevalainen, H.; Suominen, P.; Taimisto, K. *J. Biotechnol.* **1994**, *6*, 534–537.
17. Nelson, N. *J. Biol. Chem.* **1944**, *153*, 375–380.
18. Sturgeon, R.J. In: Dey, P.M.; Harborne, J.B. (Eds.) *Methods in Plant Biochemistry*, Acedemic Press, London, 1990; vol. 2, pp. 1–37.
19. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
20. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
21. van Houdenhoven, F.A.E., **1975**, Ph.D. thesis, Wageningen Agricultural University.
22. O'Neill, M. A.; Albersheim, P.; Darvill, A. G. In *Methods in Plant Biochemistry*, *Carbohydrates*; Dey, P. M., Ed.; Academic Press: London, 1990; Vol. 2, p 415.
23. Korner, R.; Limberg, G.; Mikkelsen, J. D.; Roepstorff, P. *J. Mass Spectrom.* **1998**, *33*, 836–842.
24. Benen, J. A. E.; Kester, H. C. M.; Visser, J. *Eur. J. Biochem* **1999**, *259*, 577–585.
25. Parenicova, L.; Benen, J. A. E.; Kester, H. C. M.; Visser, J. *Biochem. J.* **2000**, *345*, 637–644.
26. Walton, J. D.; Cervone, F. *Physiol. Mol. Plant Pathol.* **1990**, *36*, 351–359.
27. Voragen, A. G. J.; Pilnik, W.; Thibault, J.-F.; Axelos, M. V. A.; Renard, C. M. G. C. In *Food Polysaccharides and Their Applications*; Stephen, A. M., Ed.; Marcel Dekker: New York, 1995; pp 287–339.
28. Martinez, M. J.; Alconada, M. T.; Guillen, F.; Vazquez, C.; Reyes, F. *FEMS Microbiol. Lett.* **1991**, *81*, 145–150.
29. Riou, C.; Freyssinet, G.; Fevre, M. *Appl. Environ. Microbiol.* **1992**, *58*, 578–583.
30. Artes, P. E.; Tena, M. *Physiol. Mol. Plant Pathol.* **1990**, *37*, 107–124.
31. Brathe, J. P.; Cantenys, D.; Touze, A. *Phytopathol. Z.* **1981**, *100*, 162–171.
32. Gao, S.; Shain, L. *Physiol. Mole. Plant Pathol.* **1994**, *45*, 169–179.
33. Niture, S. K.; Pant, A.; Kumar, A. R. *Eur. J. Biochem.* **2001**, *268*, 832–840.
34. Devi, N. A.; Rao, A. G.; Appu Rao, A. G. *Enzyme Micro. Technol.* **1996**, *18*, 59–65.
35. Rexova-Benkova, L.; Markovic, O. *Adv. Carbohydr. Chem. Biochem.* **1976**, *33*, 323–385.
36. Archer, S. A. *J. Sci. Food Agric.* **1979**, *30*, 692–703.
37. Blanco, P.; Sieiro, C.; Villa, T. G. *FEMS Microbiol. Lett.* **1999**, *175*, 1–9.
38. Barnby, F. M.; Morpeth, F. F.; Pyle, D. L. *Enzyme Microb. Technol.* **1990**, *12*, 891–897.
39. Blanco, P. **1997**, Ph.D. Thesis, University of Santiago de Compostela, Spain.
40. Hasui, Y.; Fukui, Y.; Kikuchi, J.; Kato, N.; Miyairi, K.; Okuno, T. *Biosci. Biotech. Biochem.* **1998**, *62*, 852–857.
41. Kohn, O.; Markovic, E.; Machova, E. *Collect. Czech. Chem. Commun.* **1983**, *48*, 790–797.
42. Limberg, G.; Korner, R.; Buchholt, H. C.; Christensen, T. M. I. E.; Roepstorff, P.; Mikkelsen, J. D. *Carbohydr. Res.* **2000**, *327*, 293–307.