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Purification and properties of an exo-(1 → 3)-β-D-galactanase from *Aspergillus niger*

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Abstract

An exo-(1 → 3)-β-D-galactanase was purified by six chromatographic steps from a culture supernatant of *Aspergillus niger*. Its apparent molecular mass was 66 kDa, as estimated by SDS-PAGE analysis. The purified enzyme had no detectable activity on various *p*-nitrophenyl glycosides and on native plant polysaccharides but exhibited a high activity on a (1 → 3)-β-D-linked galactan backbone obtained after partial acid hydrolysis and two Smith degradations of gum arabic. The optimum conditions were pH 4.5 and 40–50°C. The enzyme had a Michaelis constant (K_m) of 1.9 mg/mL for the β-(1 → 3)-D-galactan with a maximum reaction velocity (V_{max}) of 1380 nkat/mg. The study of the reaction products obtained after enzyme treatment of two galactans derived from gum arabic through one or two Smith degradations showed that it was an exo-(1 → 3)-β-D-galactanase able to by-pass the branching points of galactan backbones and thus to release the side-chains of type II arabinogalactans in an undegraded form.

Keywords: Galactanase; Arabinogalactan; Galactan; Polysaccharide hydrolase; *Aspergillus niger*

1. Introduction

Type II arabinogalactan-proteins (AGPs) are proteoglycans widely distributed in the extracellular matrix from both higher and lower plant species [1–3]. They contain from 2 to 10% protein by weight with an amino acid composition that is characteristically rich in Ser, Hyp, and Ala. The carbohydrate moiety consists of a (1 → 3)-β-D-galactan

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backbone with side branches of (1 → 6)-linked β-D-galactosyl residues that are heavily substituted by α-L-arabinofuranosyl residues and minor amounts of glucuronic acid, rhamnose, xylose, and fucose. However, the spatial arrangement of AGPs is not fully understood and chemical methods (partial acid hydrolysis, Smith degradation, methylation analysis) are not sufficient to give new information about their overall organization.

Use of specific enzymes is now required for a better knowledge of the distribution and mode of organization of the side-chains on the (1 → 3)-β-D-galactan backbone. Purified arabinofuranosidases, galactosidases, and galactanases have to be used for a complete degradation of the carbohydrate moiety of AGPs.

α-L-Arabinofuranosidases (EC 3.2.1.55) from fungi or plants have been used to remove the terminal arabinosyl residues of AGPs [4–10], releasing the galactan core that is susceptible to further degradation by galactosidases and galactanases.

An endo-galactanase (EC 3.2.1.90) was partially purified from a culture supernatant from *Rhizopus niveus* [11]. It degraded coffee bean arabinogalactan to mixed oligomers of D-Gal and L-Ara [12]; however, this enzyme also liberated arabinose and was probably contaminated by an arabinofuranosidase.

Endo-(1 → 4)-β-D-galactanases (EC 3.2.1.89) were isolated from various sources (bacteria and fungi) but are inactive on type II AGPs [13–16].

An exo-galactanase from radish seeds, which was active on β-(1 → 3)- and β-(1 → 6)-galactooligomers, partially degraded the 1,6-linked galactan chains of dearabinosylated radish AGPs releasing galactose as the main reaction product but also some oligomers with substituted galactosyl units [17]. Synergistic action of this enzyme with an α-L-arabinofuranosidase from the same source led to 60% degradation of the carbohydrate moiety of radish seed AGP [9].

A galactanase from *Irpex lacteus* was reported as acting on radish AGPs [4,5], but complete purification and characterization were not described. An exo-(1 → 3)-β-D-galactanase has been isolated from the same source. It released D-galactose from the non-reducing termini of (1 → 3)-β-D-galactooligomers and (1 → 3)-β-D-galactans, and exhibited a particular mode of action on dearabinosylated AGPs since it was able to by-pass branching points and thus to liberate intact β-(1 → 6)-linked side-chains carrying their arabinosyl or glucuronosyl substituents and an additional galactosyl unit [18].

An endo-(1 → 6)-β-D-galactanase was isolated in our laboratory from a culture supernatant of *Aspergillus niger* [19] which specifically catalysed the hydrolysis of β-(1 → 6)-linked galactan chains releasing (1 → 6)-β-D-galactobiose and galactose in a molar ratio of 1 : 0.26. On dearabinosylated grape and wine AGPs, its action shortened the (1 → 6)-β-D-galactan chains and removed 50% of the galactose content, 3-linked galactan chains being untouched [7,20]. The detection of galactooligomers up to dp 7 in the early stage of the reaction indicated that the dp of (1 → 6)-linked side chains could be as high as 7.

No endo-(1 → 3)-β-D-galactanase active on the galactan core of AGPs has been reported to the best of our knowledge.

In the present work, we describe the purification of an exo-(1 → 3)-β-D-galactanase isolated from a culture supernatant of *A. niger*. Its mode of action on gum arabic-derived galactans was studied and found to be very similar to that reported for the exo-galactanase from *Irpex lacteus* [18].

2. Experimental

Source of enzyme.—A crude liquid enzyme preparation from *A. niger* (Arabanase 8001/8002) provided by Gist-Brocades France S.A. (Seclin, France) was used as the source of galactanase.

Substrates.—Gum arabic from *Acacia senegal* (Sigma, St Louis, USA) was partially acid hydrolysed in 20 mM trifluoroacetic acid (TFA) for 6 h at 100°C yielding a degraded product (designated as Galactan I) which was submitted to two successive Smith degradation [7] yielding Galactans II and III.

Analytical methods.—Protein content was measured by the Lowry method [21] using ovalbumin as a standard.

Arabinogalactans and galactans were methylated as described [10]. Prior to methylation, oligosaccharides (0.1–0.5 mg) were reduced [22,23] with 10 mg $NaBD_4$ (Merck, Germany) in 1 M NH_4OH (1 mL) for 1 h at room temperature, quenched with glacial acetic acid, followed by co-evaporation with acidified methanol. The residue was dissolved in water, passed through a 2-mL mixed-bed resin column of AG 50-1 X8 (H^+, OH^-; Bio-Rad, USA), freeze-dried, and methylated.

Partially methylated alditol acetates were analysed as described [24], their identities being confirmed by GC–MS performed with a HP-5989 MS-Engine (Hewlett–Packard, USA).

The response factor of the galactitol pentamethyl derivative from reduced oligosaccharides was calculated by reduction and methylation of commercial (1 → 6)-β-D-galactobiose (Sigma, USA).

Enzyme purification.—The exo-(1 → 3)-β-D-galactanase was purified from the *A. niger* liquid preparation by the following procedure. At each step, fractions able to degrade Galactan III derived from gum arabic were pooled.

Step 1. An aliquot (10 mL) from the crude *A. niger* liquid preparation was precipitated by 5.05 g of ammonium sulfate.

Step 2. The pellet was redissolved in 5 mL of 0.05 M phosphate buffer (pH 7.2) containing 1 M ammonium sulfate and loaded at 60 mL/h on a 5 × 5 cm Phenyl-Sepharose CL-4B (Pharmacia, Sweden) column equilibrated in the same buffer.

Step 3. The unbound fraction (100 mL) was dialysed against 0.025 M acetate buffer (pH 4.0) and applied at 60 mL/h on a 5 × 7.5 cm DEAE-Sephacel (Pharmacia) column equilibrated in the same buffer. Proteins were eluted with a 0 → 0.4 M NaCl gradient.

Step 4. The fraction eluted at 0.05 M NaCl (38 mL) was dialysed against 0.02 M phosphate buffer (pH 7.0) and applied at 60 mL/h on a 0.6 × 10 cm D Zephyr (Sepracor-IBF, France) column equilibrated in the same buffer. Proteins were eluted with a 0 → 0.4 M NaCl gradient.

Step 5. Fractions eluted at 0.25 M NaCl were pooled (80 mL), dialysed against 0.05 M phosphate buffer (pH 4.8), and applied at 60 mL/h on a D Zephyr column equilibrated in the same buffer. Proteins were eluted with a 0 → 0.4 M NaCl gradient.

Step 6. Fractions eluted at 0.1 M NaCl were pooled (4 mL) and loaded at 12 mL/h on a 1 × 100 cm Ultrogel AcA 44 (Sepracor-IBF) column equilibrated in 0.025 M acetate buffer (pH 4.2).

Step 7. The main protein peak (10 mL) was dialysed against 0.025 M acetate buffer

(pH 3.6) and injected at 60 mL/h onto a 0.6 × 10 cm S Zephyr (Sepracor-IBF) column equilibrated in the same buffer. A 0 → 0.1 M NaCl gradient allowed elution of the purified enzyme.

Enzyme assays.—During enzyme purification, the ability of fractions to degrade Galactan III was checked by high performance size exclusion chromatography (HPSEC) on two serial Shodex OHpak KB-803 and KB-805 columns (30 × 0.8 cm; Showa Denkko, Japan) with a OHpak KB-800P guard column (5 × 0.6 cm), equilibrated at 1 mL/min with 0.1 M $LiNO_3$ [25], the elution being followed by refractometry.

The exo-(1 → 3)-β-D-galactanase activity was measured by measuring the liberation of galactose by β-D-galactose dehydrogenase from *Pseudomonas fluorescens* [26], an enzyme known to oxidize galactose but also galactobiose yielding 1 mol of NADH per mol of galactobiose [17]. The reaction was performed by incubating 25 μL of a 5 mg/mL Galactan III solution in 30 mM acetate buffer (pH 4.2) and 5 μL of enzyme (< 0.1 nkat) at 40°C for 20 min and stopped by boiling the sample for 5 min. 850 μL of 0.1 M Tris-HCl buffer (pH 8.6), 100 μL of 5 mM NAD^+ solution in water, and 20 μL of β-D-galactose dehydrogenase (Boehringer Mannheim, Germany) were then added to the mixture, which was kept at room temperature for 90 min. Absorbance at 340 nm was measured and the activity expressed in nkat.

Glycosidase activities [α-D-glucosidase (EC 3.2.1.20), β-D-glucosidase (EC 3.2.1.21), β-D-mannosidase (EC 3.2.1.25), β-D-galactosidase (EC 3.2.1.23), and α-L-arabinofuranosidase (EC 3.2.1.55)] were assayed by measuring the amount of *p*-nitrophenol released from 4 mM solutions of the respective *p*-nitrophenyl glycosides in 30 mM acetate buffer (pH 4.2) [25 μL incubated in the presence of 5 μL of enzyme (0.06 nkat) for 8 h at 40°C].

Other residual activities were tested by incubating 50 μL solutions (5 mg/mL) in acetate buffer of the following poly- or oligo-saccharides: carboxymethylcellulose, guar gum, oat spelts xylan, yeast mannan, dextran, polygalacturonic acid, lichenan, (1 → 6)-β-D-galactobiose (all from Sigma), potato galactan, and barley β-glucan, with 5 μL of enzyme (0.06 nkat), for 24 h at 40°C. Release of mono- or oligo-saccharides was monitored either by TLC [19] or by high pH anion-exchange chromatography (HPAEC) as described further.

Study of enzyme properties.—SDS-PAGE was performed [27] with a 5% stacking gel and a 10% separating gel, proteins being stained with Coomassie Brilliant Blue R-250. HPSEC of the purified enzyme on serial Shodex columns was performed as described in the "Enzyme assays" section, elution being followed at 280 nm.

The optimum pH value was determined by measuring enzyme activity on Galactan III solutions (5 mg/mL) in universal buffer (pH range 2.5 → 11). The optimum reaction temperature was obtained by measuring the enzyme activity on Galactan III solutions (5 mg/mL) in 30 mM acetate buffer (pH 4.2), incubated for 20 min at temperatures from 20 to 80°C. The remaining activity after pre-incubation of the enzyme at 40°C for 20 h and inhibition by D-galactonic acid lactone (Sigma) were measured under standard conditions.

Product analysis.—In order to recover reaction products, gum arabic-derived Galactans II and III (25 mg) in 30 mM acetate buffer (5 mL, pH 4.2) were incubated with 0.6 nkat of exo-galactanase at 40°C for 48 h, 0.3 nkat being added after 24 h. Aliquots were

removed and diluted 50 × in 10 mM NaOH before HPAEC analysis on a Dionex CarboPac PA-1 column (25 × 0.4 cm) with a CarboPac PA-1 guard column (5 × 0.4 cm; Dionex, USA) eluted at 1 mL/min with the following gradient of sodium acetate in 100 mM NaOH: 0–5 min, isocratic at 6 mM sodium acetate; 5–15 min, linear gradient up to 0.1 M sodium acetate; 15–25 min, linear gradient up to 0.3 M sodium acetate, and final isocratic elution with a Dionex DX-300 chromatography system equipped with a PAD detector.

The whole mixtures were loaded onto a Bio-Gel P-2 column (100 × 1.6 cm) equilibrated with water and the oligomeric products were eluted at 12 mL/h, recovered separately, and identified by HPAEC and methylation analysis. Samples of each oligosaccharide at 20 μg/mL in 0.1 mL of acetate buffer (pH 4.2) were incubated 4 h at 40°C with 0.5 nkat of β-D-galactosidase from *A. niger* (Sigma) and/or 0.6 nkat of an α-L-arabinofuranosidase purified in our laboratory [7] from the same culture supernatant of *A. niger* as used as the source of exo-galactanase in the present study.

3. Results and discussion

Characterization of substrates.—Gum arabic was submitted to partial acid hydrolysis and provided Galactan I (yield 53%). Two Smith degradations of Galactan I led to Galactans II and III (respective yields, 14 and 4%). The methylation data obtained for native gum and degraded products are given in Table 1. The (terminal/branched residue) ratios obtained for both native gum and partially hydrolysed Galactan I were low, probably because of the presence of terminal glucosyluronic acid groups [28] that were not quantified in the present study. Galactan II was composed mainly of 3- and 3,6-linked galactose but still contained some terminal arabinose. The final product, Galactan III, was mainly a (1 → 3)-linked galactan with interlinkages at position 6, a

Table 1
Methylation analysis of native gum arabic and derived galactans (relative mol %)

Methyl ether	Linkage	Native gum	Galactan I	Galactan II	Galactan III
2,3,4-Rha [a]	Terminal	10.7	8.2		
3,4-Rha	1 → 2	1.1	[b]		
2,3,5-Ara	Terminal	16.3	8.9	10.6	
2,3,4-Ara	Terminal	1.7			
2,5-Ara	1 → 3	15.0	8.7		
3,5-Ara	1 → 2	1.7			
2-Ara	1 → 3,5	2.0			
2,3,4,6-Gal	Terminal	0.7	6.1	11.2	23.3
2,4,6-Gal	1 → 3	3.0	4.6	47.6	51.5
2,3,4-Gal	1 → 6	1.7	19.4	4.1	2.6
2,6-Gal	1 → 3,4	5.1	3.1	2.5	3.6
2,4-Gal	1 → 3,6	26.4	37.3	21.4	16.3
2-Gal	1 → 3,4,6	14.6	3.7	2.6	2.7

[a] 2,3,4-Rha denotes 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol, etc.
[b] No entry indicates < 0.5% of the total.

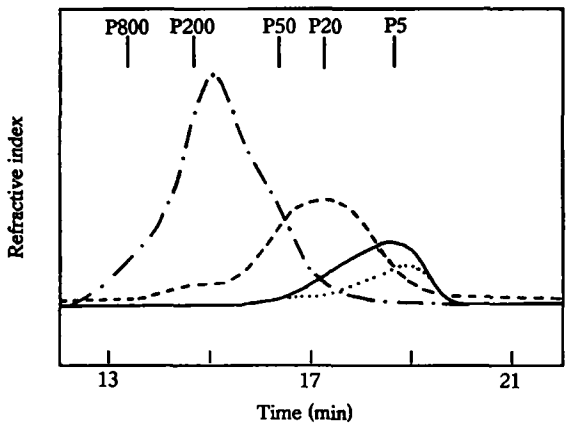

Fig. 1. HPSEC profiles of native gum arabic (·–·–·) and degraded products Galactans I (— — —), II (———), and III (· · · · · ·) on Shodex OHpak-KB columns. Positions and M_w values in kDa of the pullulan standards are indicated.

structural feature typical of the galactan core of type II AGPs [7,28]. As compared to the high molecular weight polydisperse gum arabic, Galactans II and III eluted as low molecular weight polysaccharides in HPSEC (Fig. 1).

Purification of the enzyme.—The initial chromatographic steps of the enzyme purification procedure allowed elimination of 86% of the proteins. The anion-exchange chromatography on D Zephyr at pH 7.0 was then very efficient (Fig. 2) since exo-galactanase was present in the last protein peak eluted from the column. Glycosidases were efficiently eliminated (galactosidase 95%, arabinosidase 89%) and 95% of the proteins were removed at this step.

Elution profiles on D Zephyr at pH 4.8 and S Zephyr at pH 3.6 are shown in Fig. 2. Exo-galactanase activity eluted from the S Zephyr column as a thin, symmetrical peak at 50 mM NaCl, the very last traces of glycosidases activities being eliminated in the unbound fraction.

Due to the presence of high levels of contaminant galactosidase activity, the exo-galactanase activity could only be measured in the last steps of the purification procedure (Table 2; purification factor 12). The purified enzyme was stored at 4°C, in 30 mM acetate buffer (pH 4.2) containing 0.02% NaN_3.

Enzyme purity and properties.—The purified enzyme gave one main band corresponding to an apparent molecular weight of 66 kDa, and a few additional very faint bands in SDS-PAGE, and one peak on HPSEC which eluted at the same volume as bovine albumin.

The purified enzyme had no detectable activity on *p*-nitrophenyl glycosides incubated for 8 h at 40°C, being thus totally devoid of α-L-arabinofuranosidase and β-D-galactosidase activities which might have interfered in the degradation of galactans. No release of mono- or oligo-saccharides was observed by TLC or HPAEC after 24 h incubation of the enzyme with various oligo- and poly-saccharides including potato (1 → 4)-β-D-galactan and (1 → 6)-β-D-galactobiose.

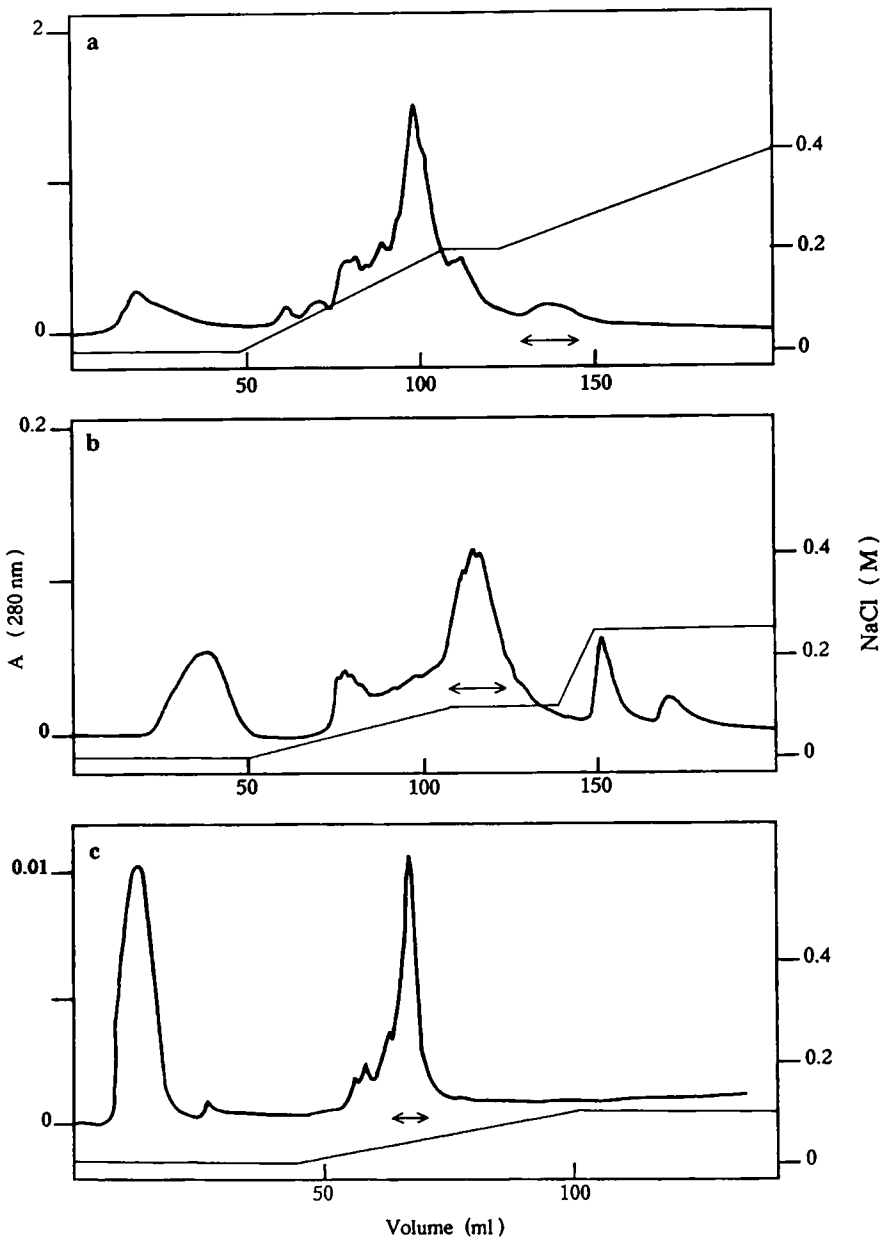

Fig. 2. Protein elution and enzyme recovery on various columns during the purification of exo-(1 → 3)-β-D-galactanase: D Zephyr pH 7.0 (a); D Zephyr pH 4.8 (b); S Zephyr pH 3.6 (c). The arrows indicated the fractions able to degrade Galactan III that were collected, NaCl gradients are indicated.

The enzyme was highly active on Galactan III with a specific activity of 760 nkat/mg measured as galactose equivalent with β-D-galactose dehydrogenase. The enzyme exhibited a typical Michaelis–Menten curve, the K_m and V_{max} for Galactan III

Table 2
Purification of exo-(1 → 3)-ß-D-galactanase from *A. niger*

Step	Protein (mg)	Activity (nkat)	Specific activity (nkat/mg)	Purification factor
Ammonium sulfate	478			
Phenyl-Sepharose CL-4B	347			
DEAE-Sephacel pH 4.0	63			
D Zephyr pH 7.0	3.2	197	60	1
D Zephyr pH 4.8	0.50	132	260	4.3
Ultrogel AcA 44	0.37	122	330	5.5
S Zephyr pH 3.6	0.10	79	760	12.7

were determined by a Hanes plot: $K_m = 1.9$ mg/mL, $V_{max} = 1380$ nkat/mg. Thus, the purified enzyme had a high specificity for (1 → 3)-β-D-linked galactan chains.

The enzyme had a maximum activity at pH 4.5 (Fig. 3) when incubated at 40°C for 20 min, the activity decreasing strongly at pH > 6. The optimum temperature was 40–50°C at pH 4.2 in a 20-min assay (Fig. 4), the enzyme retaining 40% of its activity after 20 h at 40°C. D-Galactonic acid lactone at 1 mM had a slight effect on the enzyme activity (5% inhibition), but inhibited the activity by 45% at 10 mM.

Analysis of reaction products and mode of action.—Removal of the complex side chains of native gum arabic by partial acid hydrolysis and Smith degradation rendered the derived polysaccharides susceptible to the enzyme. The relative activities were low with the native gum and Galactan I (1.2 and 4.7%, respectively) and increased with the content in (1 → 3)-β-D-linked galactose to reach with Galactan II 86.5% of the maximum activity obtained with Galactan III.

Galactans II and III were degraded for 72 h by the enzyme, with the time course of liberation of reaction products being monitored by HPAEC (final hydrolysis 21% and

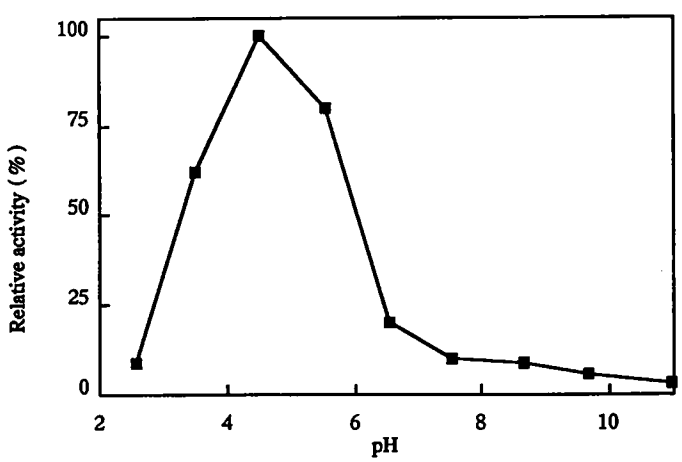

Fig. 3. Effect of pH on the activity of exo-(1 → 3)-β-D-galactanase during 20 min incubations at 40°C, the values given are the average of duplicate assays.

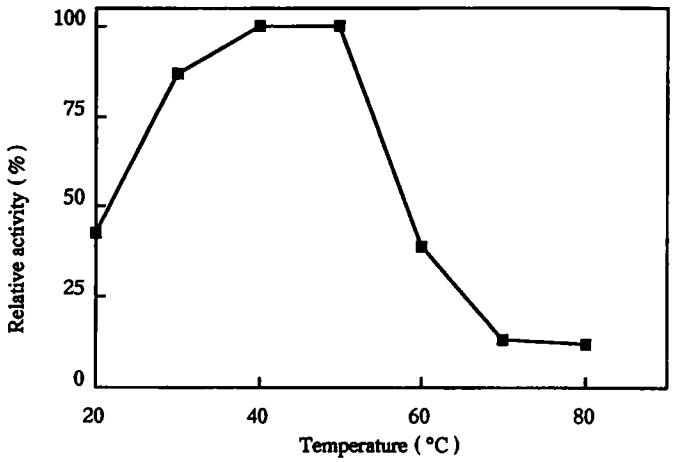

Fig. 4. Effect of temperature on the activity of exo-(1 → 3)-β-D-galactanase, incubations being performed for 20 min at pH 4.2, the values given are the average of duplicate assays.

18%, respectively). The digests were then fractionated on Bio-Gel P-2, the purity of each isolated fraction being checked by HPAEC. The composition and structure of each reaction product were determined by sugar and methylation analyses in combination with degradation by β-D-galactosidase and α-L-arabinosidase and subsequent HPAEC.

Treatment of Galactan II with the purified enzyme yielded 3 main reaction products designated as P1, P2, and P3 that were subsequently purified on Bio-Gel P-2.

P1, the main reaction product, was identified as monomeric galactose by HPAEC and GC of its alditol acetate derivative.

P2 coeluted with commercial (1 → 6)-β-D-galactobiose in HPAEC and was totally degraded by an *A. niger* β-D-galactosidase. After reduction and methylation, 1,2,3,4,5-penta- and 2,3,4,6-tetra-*O*-methyl-D-galactitol were obtained in a molar ratio of 1 : 1.1. Product P2 was thus identified as (1 → 6)-β-D-galactobiose.

P3 yielded arabinose and (1 → 6)-β-D-galactobiose in an equimolar ratio when submitted to α-L-arabinofuranosidase degradation. β-D-Galactosidase alone was inactive on P3 but its synergistic action with α-L-arabinosidase yielded arabinose and galactose in a molar ratio of 1 : 2.1. Methylation analysis after reduction of P3 afforded 1,2,3,4,5-penta- and 2,4,6-tri-*O*-methyl-D-galactitol and 2,3,5-tri-*O*-methyl-L-arabinitol in a molar ratio of 1 : 1.3 : 0.8. This product was thus identified as L-Ara*f*-α-(1 → 3)-D-Gal*p*-β-(1 → 6)-D-Gal*p*.

Traces of reaction products of higher dp were detected by HPAEC but, due to their scarcity, they could not be formally identified.

Degradation of Galactan III with the enzyme yielded two main products that were identified as galactose and (1 → 6)-β-D-galactobiose.

Molar ratios of reaction products were galactose/(1 → 6)-β-D-galactobiose/L-Ara*f*-α-(1 → 3)-D-Gal*p*-β-(1 → 6)-β-Gal*p*, 1 : 0.14 : 0.25 for Galactan II and galactose–(1 → 6)-β-D-galactobiose, 1 : 0.20 for Galactan III.

These data are very similar to those reported for an exo-(1 → 3)-β-D-galactanase from *Irpex lacteus* [18], an enzyme specific for (1 → 3)-linked chains and able to by-pass the

3,6-linked branching points and thus to release the side chains of AGPs in an undegraded form carrying an additional galactosyl unit. Comparison of the molar ratios of reaction products and methylation analyses of both Galactans II and III were consistent with this mode of action: the ratios of 3,6- to (terminal + 3-linked) galactose being similar to that of (galactobiose + L-Ara*f*-α-(1 → 3)-D-Gal*p*-β-(1 → 6)-D-Gal*p*) to monomeric galactose released through enzyme action.

4. Conclusions

This paper is the first report of the isolation and characterization of an exo-(1 → 3)-β-D-galactanase from *A. niger*, the main source of enzyme used in the food industry. An enzyme with a similar mode of action has been previously isolated from *I. lacteus* [18].

The peculiar mode of action of this enzyme, which degraded the (1 → 3)-linked galactan chains of the backbone of type II arabinogalactans and released the (1 → 6)-linked chains carrying an additional galactosyl unit, and the absence of contaminating glycosidase activities makes our preparation a useful tool for the study of the side-chains of type II AGs and AGPs. Study of the action of this enzyme on various AGPs is now in progress.

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