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Purification and characterisation of a β-galactosidase from *Aspergillus aculeatus* with activity towards (modified) exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* B39 and B891

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Abstract

β-Galactosidase from *Aspergillus aculeatus* was purified from a commercial source for its hydrolytic activity towards (modified) exopolysaccharides (EPSs) produced by *Lactococcus lactis* subsp. *cremoris* B39 and B891. The enzyme had a molecular mass of approximately 120 kDa, a pI between 5.3 and 5.7 and was optimally active at pH 5.4 and 55–60 °C. Based on the N-terminal amino acid sequence, the enzyme probably belongs to family 35 of the glycosyl hydrolases. The catalytic mechanism was shown to be retaining and transglycosylation products were demonstrated using lactose as a substrate. The β-galactosidase was also characterised using its activity towards two EPSs having lactosyl side chains attached to different backbone structures. The enzyme degraded O-deacetylated EPS B891 faster than EPS B39. Furthermore, the presence of acetyl groups in EPS B891 slowed down the hydrolysing rate, but the enzyme was still able to release all terminally linked galactose.

Keywords: β-Galactosidase; *Aspergillus aculeatus*; Exopolysaccharide; *Lactococcus lactis* subsp. *cremoris*; Enzymic modification

1. Introduction

Enzymes can be very useful in polysaccharide research for at least two reasons. Firstly, they can be helpful during structure elucidation. Secondly, they can be used to modify the chemical structure of polysaccharides in order to alter their physical properties. Relatively few polysaccharases with activity towards microbial exopolysaccharides (EPSs) have been isolated and characterised [1]. Most enzymes active on these polysaccharides are highly specific and seldom act on more than one substrate, unless the structures are very similar [2]. Since there are very few commercially available enzymes acting on EPSs, the laboratory must normally isolate its own enzymes [1].

For these reasons, many crude enzyme preparations were screened for both endo- and exo-activity towards EPSs produced by *Lactococcus lactis* subsp. *cremoris* B40 [3,4], B39 [5] and B891 [6]. It appeared to be extremely

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difficult to find enzyme preparations able to degrade these EPSs and only a few preparations showed some activity towards these (chemically modified) polysaccharides. Interestingly, a crude enzyme preparation from *Aspergillus aculeatus* was shown to contain one or more β-galactosidases able to release galactose from EPS B39 [5] and from EPS B891 (after O-deacetylation) [6]. For both polysaccharides, the enzyme preparation was very useful in determining the structure of the side chains. Knowing the chemical structures of EPS B39 and EPS B891 (Fig. 1) and noticing the similarities between the side chains, the activity towards both substrates can probably be attributed to one β-galactosidase in this enzyme preparation.

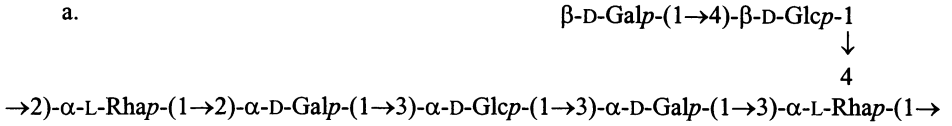
In the present study, we report the purification and characterisation of this β-galactosidase (E.C. 3.2.1.23) from *A. aculeatus* and its action towards several (chemically modified) EPSs.

2. Experimental

Substrates.—The origin and chemical structures of EPSs produced by *L. lactis* subsp. *cremoris* EPS B39 and EPS B891 have been described before [5,6]. For both EPSs, two grades of purity were used: (I) Partially purified EPS was obtained by selective extraction/precipitation using CCl_3CO_2H and EtOH. (II) Purified EPS was obtained from the partially purified EPS by size-exclusion chromatography. (Partially) purified EPS B891 was O-deacetylated as described [6]. *p*-Nitrophenyl β-D-galactopyranoside (*p*np-β-D-Gal*p*) was obtained from Koch and Light Ltd. (Haverhill, England), *p*-nitrophenyl β-D-fucopyranoside (*p*np-β-D-Fuc*p*) was obtained from Sigma (St. Louis, USA), and lactose was obtained from E. Merck (Darmstadt, Germany).

Enzyme purification.—β-Galactosidase was purified from the commercial enzyme preparation Pectinex Ultra SP produced by *A. aculeatus* (Novo Nordisk Ferment AG, Dittingen, Switzerland) starting from 60 mL. Purification involved desalting on Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, CA), followed by separation on DEAE Sepharose Fast Flow, MonoS HR 5/5 and Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden). Experimental details are given in Fig. 2. Separation on the Bio-Gel P-10 column was carried out at 4 °C, whereas the other purification steps were performed at 20 °C. All buffers contained 0.01% (w/v) NaN_3 to prevent microbial growth, except for the buffers used for the DEAE Sepharose column. Therefore, NaN_3 was directly added to the fractions obtained after this separation step. In each purification step, the protein content (A_{280}) was monitored and the fractions were screened for β-galactosidase activity towards *p*np-β-D-Gal*p*. Fractions containing activity were also screened towards partially purified O-deacetylated EPS B891 and the fractions having the highest activity were pooled. The

a.



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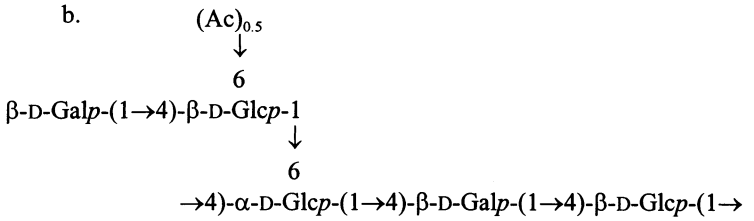

Fig. 1. Chemical structures of the repeating units of the EPSs produced by *L. lactis* subsp. *cremoris* B39 (a) [5] and B891 (b) [6]. The approximate relative amount of acetyl groups present (100% = 1) is given between brackets.

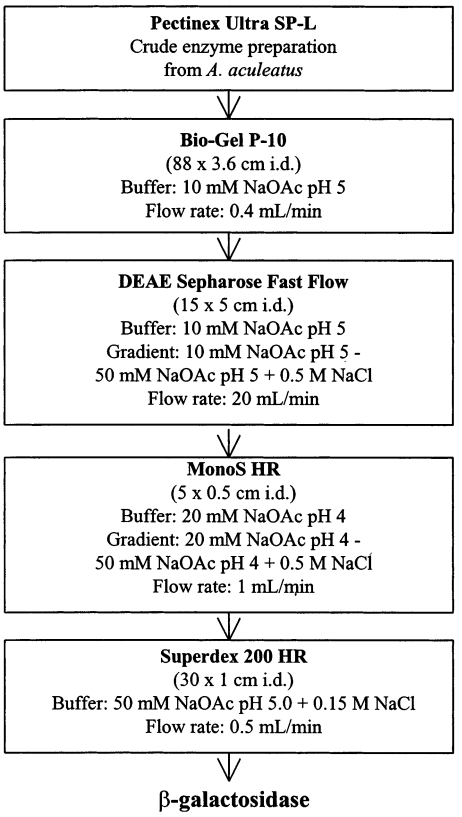

Fig. 2. Purification scheme of β-galactosidase from Pectinex Ultra SP produced by *A. aculeatus*.

resulting pool after separation on the DEAE column was dialysed against 20 mM NaOAc pH 4 (4 °C) before further purification on the MonoS column. After the last two separation steps, the active fractions were pooled according to their purity as judged from native polyacrylamide gel electrophoresis (PAGE). The resulting pool after separation on Superdex 200 was dialysed against 50 mM NaOAc pH 5 (4 °C) and characterisation of the β-galactosidase was performed using this pool.

Enzyme assays.—Column fractions containing activity towards *p*np-β-D-Gal*p* were screened for activity towards partially purified O-deacetylated EPS B891. The release of galactose was determined from the formation of NADH from NAD^+ after addition of β-galactose dehydrogenase S (Roche Molecular Biochemicals, F. Hoffmann-La Roche Ltd., Basel, Switzerland). This fast method, based on the method described by Sturgeon [7], was used to pool the collected fractions properly and no enzyme activities were calculated from these data.

β-Galactosidase activity towards EPS was calculated from the release of galactose as determined by high-performance anion-exchange chromatography (HPAEC) [3], whereas the release of *p*-nitrophenol from *p*np-β-D-Gal*p* was measured spectrophotometrically at 405 nm and activity was calculated using the molar extinction coefficient of 13,700 M^{-1} cm^{-1}. The substrate concentration was 0.02% (w/v) for *p*np-β-D-Gal*p* (and *p*np-β-D-Fuc*p*) and 0.16% (w/v) for partially purified (O-deacetylated) EPS. One unit of enzyme activity (U) is defined as the amount of enzyme that liberates 1 μmol galactose per min (in 50 mM NaOAc pH 5.0 at 30 °C).

Determination of protein content.—The protein content was determined according to the procedure of Bradford [8] using bovine serum albumin as a standard.

Determination of temperature- and pH optimum.—The substrate concentration was 0.02% (w/v) of *p*np-β-D-Gal*p* and incubations took place for 1 h at 30 °C in 50 mM NaOAc pH 5 unless mentioned otherwise. The optimum temperature for the β-galactosidase was determined by incubating at temperatures in the range 0–70 °C. The optimum pH was determined using McIlvaine buffers (mixtures of 0.08 M citric acid and 0.16 M sodium hydrogenphosphate) in the pH range 3–7.5. The protein concentration was 0.065 μg/mL for the incubations in NaOAc buffer and 0.26 μg/mL for the incubations in McIlvaine buffer, because of the lower enzyme activity in the latter buffer.

Determination of molecular mass and pI.—The molecular mass was determined using three methods: (I) by PAGE, using a PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the instructions of the supplier. The molecular mass was estimated by SDS-PAGE on a PhastGel gradient 10–15 gel, using a low molecular mass kit from 14.4 to 94.0 kDa for calibration. The proteins were silver stained. (II) By determination from the Superdex 200 gel filtration chromatography column (Fig. 2). Calibration was performed using ribonuclease A (13.7 kDa), chymotrypsin A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa) and ferritin

(440 kDa). (III) By matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS), using a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, USA) in the positive mode. The enzyme was concentrated and desalted in a Vivaspin 500 μL concentrator (5000 MWCO, Vivascience, Inc., Binbrook Hill, UK) and then mixed (1 μL) with 1 μL matrix (10 mg 2,5-dihydroxybenzoic acid in 1 mL distilled water). The mass spectrometer was used in the linear mode and calibrated with bovine serum albumin ($[M+H]^+ = 66{,}431$).

The pI was deduced from a pH 4–6.5 isoelectric focusing gel using a PhastSystem and the low pI calibration kit (Amersham Pharmacia Biotech). The proteins were silver stained or the activity was visualised under UV light by soaking the gel in 10 mM NaOAc pH 5 + 0.01% NaN_3 containing 1 mM 4-methylumbelliferyl-β-D-galactoside.

Deglycosylation of β-galactosidase.—Deglycosylation of the enzyme with *N*-glycosidase F (Roche Molecular Biochemicals, F. Hoffmann-La Roche Ltd.) was performed according to the instructions of the Glycopro deglycosylation kit (ProZyme, Inc., San Leandro, USA). Analysis was performed by SDS-PAGE (vide supra).

Determination of N-terminal amino acid sequence.—The N-terminal amino acid sequence was determined after SDS-PAGE followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane by the Sequence Centre of Utrecht University (The Netherlands). Analysis was performed, using automated Edman degradation, on a Perkin–Elmer Applied Biosystems Protein Sequencer 476A.

Kinetics of β-galactosidase towards p*np-β*-D-*Gal*p.—*p*np-β-D-Gal*p* was incubated with β-galactosidase (in 50 mM NaOAc pH 5.0, 30 °C, 1 h) at substrate concentrations in the range 0.008 mM (0.0002% w/v) to 8 mM (0.24% w/v). The inhibitory effect of D-galactose on β-galactosidase activity was investigated using different concentrations of *p*np-β-D-Gal*p* (0.015–6 mM) and D-galactose (0–10 mM). The protein concentration was 0.065 μg/mL.

Kinetics of β-galactosidase towards lactose.—Lactose was incubated with β-galactosidase (in 50 mM NaOAc pH 5.0, 30 °C, 1 h) with substrate concentrations in the range 0.08–80 mM. The protein concentration was 0.52 μg/mL. The β-galactosidase activity was calculated from the release of galactose as determined by HPAEC [3]. Transglycosylation products were determined by HPAEC [9] and MALDI-TOF MS [4] using a substrate concentration of 80 mM.

Stereochemical course of hydrolysis.—Substrate (0.2 mg *p*np-β-D-Gal*p* in 0.5 mL 50 mM NaOAc buffer, pH 5) and enzyme (0.16 mg β-galactosidase in H_2O) were freeze dried three times from D_2O (99.9 atom% D, Cambridge Isotope Laboratories, USA) to exchange labile ^1H atoms for D. Just prior to ^1H NMR analysis, the substrate was dissolved in 0.7 mL 99.96% D_2O (Cambridge Isotope Laboratories) and the pD adjusted to approximately 5 by the addition of 1% DCl. The solution was then equilibrated at 30 °C in a 5 mm NMR tube and the initial spectrum was recorded on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. The enzyme was dissolved in 100 μl 99.96% D_2O and 20 μL of this solution was added to the NMR tube. The stereochemical course of hydrolysis was followed by recording ^1H NMR spectra at intervals during the incubation. Afterwards, a trace of acetone was added to the NMR tube and a final spectrum was recorded for calibration (δ 2.225 ppm).

Activity of β-galactosidase towards purified EPSs.—Purified EPS B39, B891 and O-deacetylated B891 were dissolved in 50 mM NaOAc pH 5.0 containing 0.01% (w/v) NaN_3 in a way that the resulting concentration of terminally linked galactose (ca. 160 μg/mL) was similar for all substrates. The substrates were incubated (30 °C) with β-galactosidase (protein concentration was 18 μg/mL) and the enzyme was inactivated at set time intervals by heating at 100 °C for 10 min. The release of galactose was analysed by HPAEC [3].

To verify the absence of galactose in enzyme-treated EPS B39 and EPS B891 and the presence of acetyl groups in EPS B891 after incubation with β-galactosidase, incubations were repeated on a larger scale as described

above. After 32 h of incubation, no heat treatment was given to inactivate the enzyme, because acetyl groups are heat-unstable [10]. The digests were dialysed against distilled water and freeze dried three times from D_2O (99.9 atom% D, Cambridge Isotope Laboratories). The substrates were dissolved in 0.7 mL 99.96% D_2O (Cambridge Isotope Laboratories) and analysed by ^1H NMR at 80 °C in a 5 mm NMR tube on a Bruker AMX-500 spectrometer.

3. Results

Purification of β-galactosidase.—The purification scheme of β-galactosidase is given in Fig. 2 and the results are summarised in Table 1. The overall purification of the enzyme on protein basis was approximately 15-fold and the overall yield of β-galactosidase activity was ca. 15%. The specific activity of the purified β-galactosidase towards *p*np-β-D-Gal*p* was 24 U/mg, which was lower than reported for purified β-galactosidase from *Aspergillus niger* (49 U/mg [11] and 33 U/mg [12]). Furthermore, it was obvious (Table 1) that the activity towards *p*np-β-D-Gal*p* was much higher (× 333) than the activity towards O-deacetylated EPS B891.

Characterisation of β-galactosidase.—The molecular mass of β-galactosidase estimated by size-exclusion chromatography was approximately 130 kDa. SDS-PAGE revealed a mass of 110 kDa, indicating that the enzyme is monomeric. By using MALDI-TOF MS, three ions were found at m/z of approximately 120,000 $[M+H]^+$, 60,000 $[M+2H]^+$, and 40,000 $[M+3H]^+$, confirming that the molecular mass of β-galactosidase is ca. 120 kDa. Similar molecular masses have been reported for β-galactosidase from *A. niger* [11–13], *Aspergillus oryzae* [14,15], *Aspergillus fonsecaeus* [16] and *Aspergillus foetidus* [17]. Upon deglycosylation, the molecular mass found by SDS-PAGE decreased slightly. This indicates that, like most fungal glycosidases [12], the enzyme is a glycoprotein.

The isoelectric point (pI) of the β-galactosidase was determined by isoelectric focusing and 3 × 2 major bands were found after silver staining with pI values in the range 5.3–5.7. When using 4-methylumbelliferyl-β-D-galactoside for enzyme activity screening, the area with the six bands was visualised under UV light. It appeared that each group with two bands contains β-galactosidase activity and that multiple forms of the β-galactosidase are present. Differences in chromatographic behaviour and physico-chemical characteristics such as pI values are often observed for fungal extracellular enzymes [12], and no further attempts were made to investigate the presence of multiple forms. The pI values reported here are higher than those reported for β-galactosidases from other Aspergilli (4.2–4.9) [11–13,15,16].

The optimum temperature of β-galactosidase in NaOAc buffer was 55–60 °C. This temperature optimum varies from the optima reported for β-galactosidases produced by *A. niger* (60–65 °C) [12], *A. oryzae* (45–55 °C) [14,15,18], *A. foetidus* (67 °C) [17] and *Aspergillus phoenicis* (70 °C) [19]. The optimum pH was found at pH 5.4 in McIlvaine buffers and was higher than the optima reported for the

Table 1
Purification of β-galactosidase from *A. aculeatus*

	*p*np-β-D-Gal*p*		O-deacetylated EPS B891	
Purification step	Specific activity (U/mg)	Yield [a] (%)	Specific activity (mU/mg)	Yield [a] (%)
Bio-Gel P-10	2	100	4	100
DEAE Sepharose Fast Flow	n.d. [b]	n.d. [b]	21	64
MonoS HR	27	20	69	22
Superdex 200 HR	24	13	72	17

[a] Based on activity.
[b] Not determined.

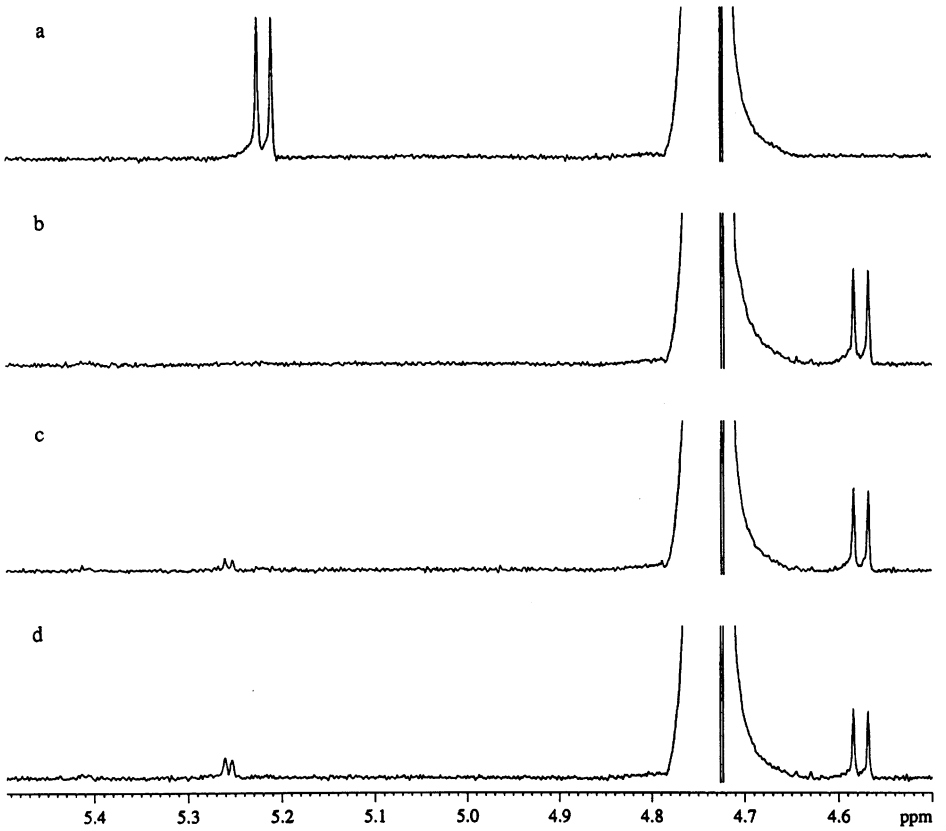

Fig. 3. Partial ^{1}H NMR spectra showing the stereochemical course of hydrolysis of *p*np-β-D-Gal*p* by *A. aculeatus* β-galactosidase: $t = 0$ min (a), $t = 7$ min (b), $t = 49$ min (c) and $t = 22.5$ h (d). The doublet in the spectrum at $t = 0$ (a) originated from *p*np-β-D-Gal*p*. The doublet at 5.256 ppm represents the anomeric proton of α-Gal*p*, and the doublet at 4.575 ppm represents the anomeric proton of β-Gal*p*.

β-galactosidases of other Aspergilli (2.5–5.0) [11,12,14–21].

The β-galactosidase was tested for its activity towards *p*np-β-D-Fuc*p*, using the same enzyme concentration as was used for *p*np-β-D-Gal*p*, but no significant activity towards the former substrate was found.

The N-terminal amino acid sequence was determined from the major band on SDS-PAGE. The resulting sequence was ?QKYVTWDDKSLFINGERN?. An amino acid sequence alignment was performed by using the BLAST programs [22]. The 18 N-terminal amino acid sequence returned a perfect match with the known β-galactosidase precursor from *A. niger* [23]. According to the SWISS-PROT Protein Sequence Data Bank [24], the *A. niger* β-galactosidase belongs to family 35 of the glycosyl hydrolases [25–27]. Since the N-terminal amino acid sequence of the enzyme from *A. aculeatus* matches with *A. niger* β-galactosidase and both enzymes are produced by Aspergilli, it was assumed that the β-galactosidase from *A. aculeatus* also belongs to family 35 of the glycosyl hydrolases.

Stereochemical course of hydrolysis.—There are two basic mechanisms of catalysis for glycosyl hydrolases, leading to overall retention or inversion of the stereochemistry at the cleavage point [28]. The stereochemical course of hydrolysis by β-galactosidase from *A. aculeatus* was followed by recording ^{1}H NMR spectra at intervals during the incubation (Fig. 3). Time $t = 0$ shows the partial spectrum of *p*np-β-D-Gal*p* before addition of β-galactosidase. After 7 min of incubation with β-galactosidase, the substrate was completely hydrolysed and the doublet ($^3J_{1,2}$ 7.9 Hz) found in the anomeric region was assigned to the anomeric proton of β-Gal (δ 4.575 ppm) [29]. Later in the incubation, mutarotation of the initially formed β anomers of Gal brought about the appearance of another doublet ($^3J_{1,2}$ 3.7 Hz) in the anomeric region, assigned to the

anomeric proton of α-Gal (δ 5.256 ppm) [29]. After 22.5 h, the relative intensities of the α and β anomer resonances were 0.28:0.72, which corresponds to the mutarotational equilibrium of Gal. These results prove that β-galactosidase from *A. aculeatus* catalysed the hydrolysis of *p*np-β-D-Gal*p* with retention of anomeric configuration. Since the β-galactosidase from *A. aculeatus* probably belongs to family 35 of the glycosyl hydrolases and the mechanism of catalysis appears to be conserved within each family [30], the mechanism for family 35 of the glycosyl hydrolases is probably retaining. Inferred from sequence similarities, this has been suggested before [31].

Kinetics of β-galactosidase.—β-Galactosidase from *A. aculeatus* catalysed the hydrolysis of *p*np-β-D-Gal*p* and lactose, and the dependence of the initial hydrolysis rate on the substrate concentration was investigated for both substrates. The Lineweaver–Burk plot, which was deduced from the release of *p*-nitrophenol (Fig. 4), indicated that the rate of hydrolysis of *p*np-β-D-Gal*p* by β-galactosidase decreased at higher substrate concentrations. In the hydrolysis of lactose, this effect was not observed at the corresponding substrate concentrations (Fig. 5(a)), although at the highest lactose concentrations (40 and 80 mM) this tendency was very slightly observed. A Lineweaver–Burk plot resembling Fig. 4(b) has been reported for an α-galactosidase from *Trichoderma reesei* [32] using *p*-nitrophenyl α-D-galactopyranoside as a substrate. In that study, the apparent hydrolysis inhibition in presence of a high substrate concentration was correlated with transglycosylation activity of the enzyme. In fact, transglycosylation probably interferes with the accurate detection of enzyme activity, leading to an apparent decrease in reaction rate. In the current study, the presence of transferase reactions at lactose concentrations > 10 mM was suggested by differences in the amounts of galactose and glucose released (Fig. 5(b)). Transglycosylation products are being formed when the enzyme transfers the galactose moiety of a

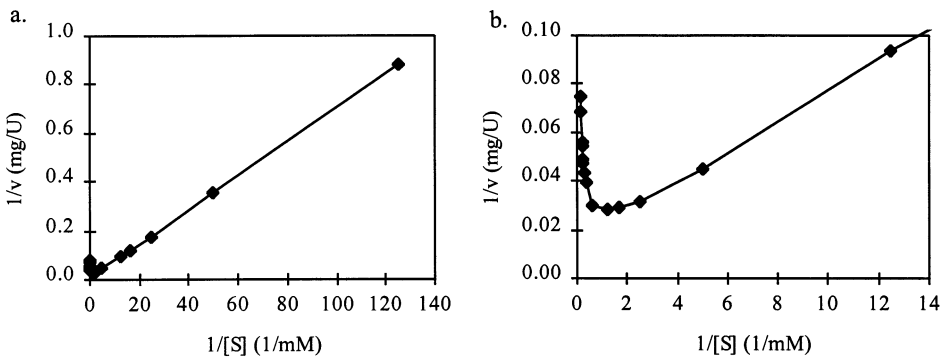

Fig. 4. Lineweaver–Burk plot for the hydrolysis of *p*np-β-D-Gal*p* catalysed by *A. aculeatus* β-galactosidase. The whole range of substrate concentration [S] is depicted in (a), whereas (b) zooms in on the area with high [S].

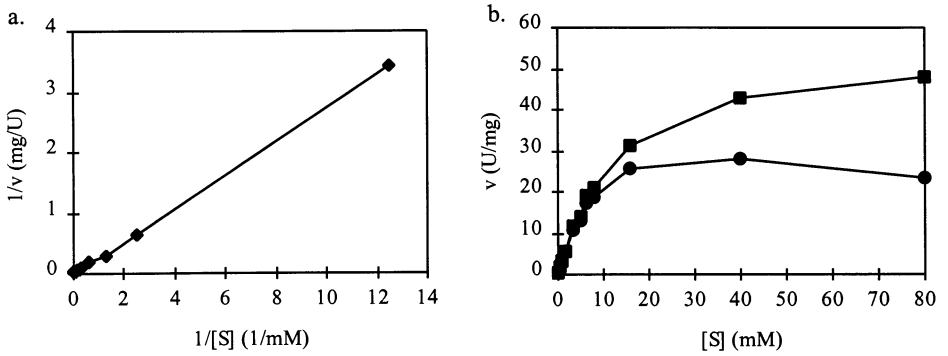

Fig. 5. Hydrolysis of lactose catalysed by *A. aculeatus* β-galactosidase: Lineweaver–Burk plot (a) and the rate of hydrolysis deduced from the glucose (■) and galactose (●) release upon substrate concentration (b).

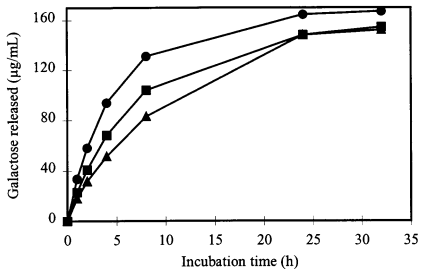

Fig. 6. Release of galactose from O-deacetylated EPS B891 (●), EPS B39 (■) and EPS B891 (▲) in time during incubation with β-galactosidase from *A. aculeatus*.

β-galactoside to an acceptor, other than water, containing a hydroxyl group [33]. Incorporation of galactose into oligosaccharides leads to different amounts of the monomeric hydrolysis products galactose and glucose as was shown for the hydrolysis of lactose by a β-galactosidase from *Streptococcus thermophilus* [34]. To investigate the formation of transglycosylation products during lactose hydrolysis at a substrate concentration of 80 mM, the digest was analysed by HPAEC and MALDI-TOF MS. Both methods proved the presence of transglycosylation products, since after incubation oligomeric peaks were found by HPAEC and a mass corresponding to a trimer of hexoses appeared in the MALDI-TOF MS spectrum (not shown). No effort was made to further characterise the obtained transglycosylation products.

The kinetic parameters K_m and k_{cat} of the reactions were evaluated from the initial rates under conditions for which the Lineweaver–Burk plot was linear. Assuming that all protein represents β-galactosidase ($M_w = 120$ kDa), an estimation of k_{cat} was derived from the obtained V_{max}. According to Fersht [35] the ratio k_{cat}/K_m is the 'specificity constant' of a substrate. For *p*np-β-D-Gal*p*, k_{cat}/K_m was $(932\ s^{-1})/(3.28\ mM) = 284\ mM\ s^{-1}$ and for lactose k_{cat}/K_m was $(254\ s^{-1})/(34.8\ mM) = 7\ mM\ s^{-1}$, indicating that the hydrolytic activity of β-galactosidase towards *p*np-β-D-Gal*p* is ca. 40 times higher than towards lactose.

D-Galactose in concentrations ≤ 1 mM did not noticeably inhibit enzymatic hydrolysis of *p*np-β-D-Gal*p*. However, 10 mM D-galactose inhibited the hydrolysis of *p*np-β-D-Gal*p* by the β-galactosidase, but only at substrate concentrations below 3 mM. These results indicate that the inhibition can be overcome by a sufficiently high substrate concentration, which means that D-galactose is a competitive inhibitor of the β-galactosidase. These results agree with the fact that galactose is generally considered to be a competitive β-galactosidase inhibitor since it competes with lactose for the substrate-binding sites in the enzyme [33].

Activity of β-galactosidase towards EPSs.— Valuable information about the substrate specificity of an enzyme can be deduced from the effects of structural modifications of a substrate on the enzymic constants k_{cat} and K_m. Ideally, these parameters should be determined for EPS B891, O-deacetylated EPS B891 and EPS B39 to investigate whether the β-galactosidase has different affinities and hydrolytic activities for the different polymers. However, the range of substrate concentration of the polysaccharides in which the reaction rate can be measured was limited due to the high viscosity and consequently no reliable values for the kinetic parameters could be obtained. Therefore, the differences in preference of the β-galactosidase for the different EPSs were investigated by measuring the release of galactose in time. Attempts were made to dissolve the EPSs in such amounts that the concentration of terminally linked galactose was equal for all samples.

The results (Fig. 6) show that the amount of galactose released after 32 h from EPS B39 (155 μg/mL) and EPS B891 (152 μg/mL) was similar and that the amount for O-deacetylated EPS B891 (167 μg/mL) was slightly higher. The addition of new enzyme after 32 h of incubation did not release more galactose (not shown) and, consequently, the end point of the reactions had been reached at that time. The higher concentration of terminally linked galactose in O-deacetylated EPS B891 might have increased the initial reaction rate. However, in another experiment (not shown), in which the initial reaction rates of β-galactosidase on O-deacetylated EPS B891 and EPS B39 were identical, the concentration of terminally linked galactose using EPS B39 was much higher (30%) than the concentration of terminally linked galactose using O-deacetylated EPS B891 as a substrate. Extrapolating these results to Fig. 6, it was concluded that

the higher initial reaction rate of O-deacetylated EPS B891 was only partially caused by the higher concentration of terminally linked galactose. Thus, at substrate concentrations with equal amounts of terminally linked galactose, O-deacetylated EPS B891 is degraded more rapidly than EPS B39, which is being degraded faster than EPS B891.

To investigate whether β-galactosidase can release all terminally linked galactose from EPS B39 and EPS B891 and to exclude the possibility that EPS B891 is slowly O-deacetylated during incubation at 30 °C, ^{1}H NMR experiments were performed on the resulting polymers of EPS B39 and EPS B891 after incubation with β-galactosidase. The results (not shown) proved that indeed all terminally linked galactose residues were released from both EPSs. Furthermore, the acetyl groups were still present in EPS B891 after incubation with β-galactosidase.

4. Discussion

Purification of the β-galactosidase activity from *A. aculeatus* resulted in one β-galactosidase, possibly consisting of multiple forms. These results conform to findings that were reported previously. Different forms of β-galactosidase were found in *A. niger* [11,13], but evidence for the existence of a β-galactosidase-encoding gene family in fungi has not yet been reported [12].

The mechanism of catalysis of β-galactosidase from *A. aculeatus* was shown to result in a retention of the anomeric configuration. According to Sinnott [28], many retaining hydrolyses contain transglycosylation activity. For β-galactosidase from *A. aculeatus* transferase activity was shown using 80 mM lactose as a substrate. Based on observations with other β-galactosidases [33,34], the concentration of transglycosylation products formed by β-galactosidase from *A. aculeatus* will probably increase at higher lactose concentrations. Using EPS as a substrate, it is unlikely that there is significant transferase activity, if any at all, since the substrate concentrations are relatively low due to the high viscosity.

The β-galactosidase from *A. aculeatus* was able to act on different EPSs with similar side chains and has been used for structural elucidation of EPSs [5,6]. Yamamoto et al. [36,37] used a β-galactosidase from Jack bean to obtain structural information about the side chains of EPS of *Lactobacillus helveticus* TY1-2 and TN-4. Like EPS B39 and O-deacetylated EPS B891, these polysaccharides contain lactosyl side chains. Therefore, it is quite possible that the β-galactosidase from *A. aculeatus* is also able to act on the EPSs from *L. helveticus* TY1-2 and TN-4. Neither the position at which the lactosyl side chain is linked to the backbone of the polymer nor the type of sugar residue in the backbone which is substituted with the lactosyl fragment, seem to be relevant for both enzymes (position 4 of α-L-Rha*p* for EPS B39, position 6 of α-D-Glc*p* for EPS B891, position 6 of β-D-Gal*p* for EPS TY1-2, and position 3 of β-D-Gal*f* for EPS TN-4).

Ester-linked substituents are widely found in bacterial EPSs [1]. With respect to EPSs produced by lactic acid bacteria, the polysaccharides produced by *Lactobacillus sake* 0-1 [38] and *L. lactis* subsp. *cremoris* B891 [6] are partially O-acetylated. According to Sutherland [2] the action of most of the polysaccharide hydrolases on EPSs is hardly effected by the presence of various acyl substituents, although the action of some polysaccharide lyases can be inhibited markedly. The present study showed that the acetyl groups did not confer total resistance to the investigated β-galactosidase and that all terminally linked galactose residues could be removed in presence of these substituents. Nevertheless, it appeared that native EPS B891 was degraded slower than O-deacetylated EPS B891. Various parameters might be involved in the explanation of this observation. Firstly, O-deacetylation of EPS might change the sample viscosity, leading to a different rate at which the enzyme encounters the substrate in solution. Under the conditions used in Fig. 6, no obvious differences in viscosity were found by visual comparison. Furthermore, the reaction rate was linearly related to the substrate concentration for all EPSs (not shown). From this, it was concluded that it is very unlikely

that the differences in reaction rates found between the EPS structures are due to differences in viscosity. Secondly, the acetyl groups present in native EPS might cause some steric hindrance for the enzyme to bind. Knowing that the acetyl groups in EPS B891 are linked to the sugar residue adjacent to the terminally linked galactose, this might very well be the case. Thirdly, the presence of acetyl groups can greatly affect the ordered structure adopted by some bacterial polysaccharides in solution [39]. Overall, the influence of the presence of acetyl substituents in EPS B891 on the hydrolysing rate of β-galactosidase seems to be directly related to the chemical structure (and ordering) of the EPS molecules. Moreover, the lower reaction rate of β-galactosidase using EPS B39 compared with O-deacetylated EPS B891 as a substrate also seems to be a matter of structure (and ordering).

In conclusion, the β-galactosidase investigated in the present study has been used for structural elucidation of EPSs [5,6], and can be used in future research to study the relationship between the chemical structure and the physical properties of these polysaccharides. The enzymic modification of EPSs might lead to improved physical properties, as has been found for a mutant type of xanthan after treatment with β-glucuronidase [40]. The structure–function relationship of EPSs modified using β-galactosidase is now under investigation.

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