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Transferase and hydrolytic activities of the laminarinase from *rhodothermus marinus* and its M133A, M133C, and M133W mutants

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Abstract Comparative studies of the transglycosylation and hydrolytic activities have been performed on the *Rhodothermus marinus* β-1,3-glucanase (laminarinase) and its M133A, M133C, and M133W mutants. The M133C mutant demonstrated near 20% greater rate of transglycosylation activity in comparison with the M133A and M133W mutants that was measured by NMR quantitation of nascent β(1-4) and β(1-6) linkages. To obtain kinetic probes for the wild-type enzyme and Met-133 mutants, *p*-nitrophenyl β-laminarin oligosaccharides of degree of polymerisation 2–8 were synthesized enzymatically. Catalytic efficiency values, k_{cat}/K_m, of the laminarinase catalysed hydrolysis of these oligosaccharides suggested possibility of four negative and at least three positive binding subsites in the active site. Comparison of action patterns of the wild-type and M133C mutant in the hydrolysis of the *p*-nitrophenyl-β-D-oligosaccharides indicated that the increased transglycosylation activity of the M133C mutant did not result from altered subsite affinities. The stereospecificity of the transglycosylation reaction also was unchanged in all mutants; the major transglycosylation products in hydrolysis of *p*-nitrophenyl laminaribioside were β-glucopyranosyl-β-1,3-D-glucopyranosyl-β-1,3-D-glucopyranose and β-glucopyranosyl-β-1,3-D-glucopyranosyl-β-1,3-D-glucpyranosyl-β-1,3-D-glucopyranoxside.

Keywords Laminarinase · *Rhodothermus marinus* · *p*-nitrophenyl β-laminarin oligosaccharides · Transglycosylation

*In a memoriam of Dr. Kirill N. Neustroev. All we, his friends and colleagues, mourn for his sudden death. He was a bright and talented scientist, brilliant manager and good friend.

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Introduction

Family 16 of the glycoside hydrolase/transglycosylase sequence classification (GH16, http://afmb.cnrs-mrs.fr/CAZY/) [1] contains enzymes with a very diverse range of substrates specificities. Agarases, carrageenanases, lichenases (β1,3−1,4–glucanases), laminarinases and xyloglucan endotransglycosylases (XETs) have so far been found in this family, part of clan B. The recently solved X-ray crystal structures of κ-carrageenanase [2], a number of β1,3−1,4−glucanases [3], two β-agarases [4], and poplar XET [5] suggest how evolutionary changes in the proteins may have brought about changes in specificity. Two groups of enzymes may be distinguished within GHF 16. Enzymes of the first group, including laminarinases and agarases,

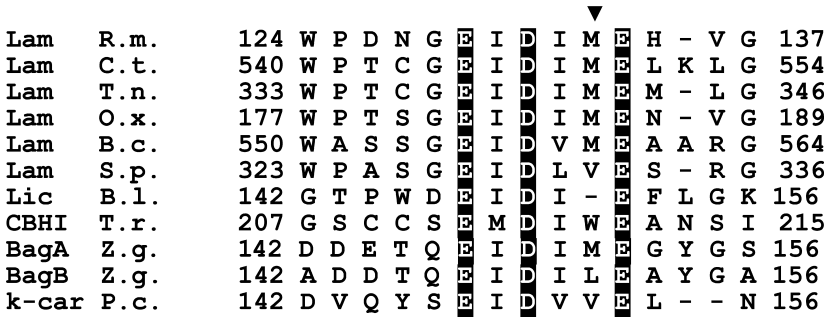


Fig. 1 Sequence comparison of the active-site region of *Rhodothermus marinus* laminarinase with several members of GH family 16 and *Trichoderma* cellobiohydrolase I. Catalytic amino acids are marked by black boxes. Invariant methionine between catalytic residues of laminarinases is marked by black triangle. Abbreviations: Lam, laminarinase; R.m., *Rhodothermus marinus;* C.t., *Clostridium thermocellum;* T.n., *Thermotoga neopolitana;* O.x., *Oerskovia xantineolitica*; B.c., *Bacillus circulans*; S.p., *Strongylocentrotus purpuratus;* Lic, lichenase; B.l. *Bacillus licheniformis*; CBHI, cellobiohydrolase I, T.r., *Trichoderma reesei*; BagA and BagB, β-agarases A and B; Z.g., *Zobellia galactanivorans;* K-car, κ-carrageenase; P.c., *Pseudoalteromonas carrageenovora*

have an active site linear sequence Glu(nucleophile)-Hph-Asp(assist)-Hph-Met-Glu(acid/base), where Hph is a hydrophobic amino acid like isoleucine. The enzymes that belong to a second group do not possess the methionine in the active site. The known X-ray structures of the GHF 16 enzymes indicate that the extra Met residue distorts a β-strand resulting in a β-bulge. Sequence alignment of the conservative region of some proteins from GHF16 argues that laminarinases are evolutionarily close to the bacterial $\beta-1,3;1,4$ glucanases of the second group, which also includes XET (Figure 1). The enzymes in Family 16 catalyze the cleavage of glycosidic bonds with overall retention of anomeric configuration, and, therefore, possess a nucleophile and an acid/base catalyst; the canonical mechanism for such enzymes is well described [6,7] previously. In Family 16 both catalytic groups were determined to be carboxylates of glutamate residues, which are close in the linear protein sequence. A catalytically important aspartate was found between them. Such "assisting" groups are found in many proteins, and their role has been variously ascribed to modulation of the ionisation states of the nucleophilic, acid/base group, and electrostatic stabilization of cationic transition states.

It was proved previously by site-directed mutagenesis of *lamR* gene, that Glu129, Asp131 and Glu134 participate in catalysis of the laminarase of *Rhodothermus marinus* [8]. This fact suggests that the presence of negatively charged side chains in vicinity of the putative nucleophile E129, the general catalytic acid E134 and the assisting residue D131 probably stabilizes the nucleophile. It was also demonstrated that deletion of M133 located near the catalytic residue E134 significantly reduced hydrolytic activity of the enzyme. Substitutions of this Met with other residues led to changes in enzymatic activities towards laminarin and β-glucan [8].

From the practical point of view, β-glucan hydrolysing enzymes, especially enzymes from thermophilic microorganisms, have industrial importance in depolymerization of mixed linked β-glucans in the brewing and animal feed-stuff industries [9]. In recent years, several successful attempts to modify transglycosylating activity of glycoside hydrolases have been reported (reviewed in 10). The most striking examples of developing genetically engineered glycosyl synthases with approximately 10-fold reduction of k_{cat} were *Agrobacterium* sp. β-glucosidase where glutamate in the active center was substituted with alanine or serine [11,12] and the *Bacillus licheniformis* 1,3–1,4-β-glucanase mutant E134A [13]. However, the necessity of using corresponding glycosyl fluorides [14] may restrict wide biotechnological exploitation of this approach. On the other hand, site-directed mutagenesis of non-catalytic residues in the active site of glycoside hydrolases can allow the use of natural substrates in transglycosylation reactions, which can be more desirable. For example, transglycosylating activities of α-amylases from *Bacillus licheniformis* and *Saccharamyces fibuligera* were improved significantly by substitution of Val with Tyr [15] and Trp with Leu [16] in the active centres of these enzymes. Finally, a hyperthermophilic *Pyrococcus furiosis* β-glucosidase mutant, which exhibited high transglycosylating ability toward lactose, serves as a successful example of the latter approach [17]. Transglycosylating ability of the β-1,3-(4)-glucanases, along with their hydrolytic properties, are of a particular interest in view of their utility in enzymatic synthesis of new β-glucooligosaccharides [18,19], artificial β-1,3-glucanase substrates [20], and branched/non-branched polyglucans, which might be used as immunostimulators in pharmacology and medicine [21,22].

In view of the above considerations, the *R. marinus* laminarinase might be a suitable vehicle for exploring the balance between hydrolysis and transglycosylation, at least in this glycosyl hydrolase family. It seems the glycosyl-enzyme intermediate interaction with water or acceptor will depend on the affinity of the $+1$ site for the acceptor, and the catalytic competence of the glycosyl-enzyme/acceptor complex. Orientation of the acid-base catalyst by correct occupancy of the $+1$ subsite may be of crucial importance in this family:

for example, the glycosyl-enzyme intermediates during XET action appear to be completely stable to hydrolysis [5]. Here we report the production of mutant *R. marinus* laminarase, in which the key Met residue producing the "β-bulge" has been changed to Ala, Cys and Trp, and describe the effects of these changes on the balance between transglycosylation and hydrolysis with this enzyme.

Materials and methods

General methods

SDS-PAGE was carried out according to the Laemmli procedure [23]. Protein concentration was measured following the Lowry [24]. Oligosaccharide substrates and products of enzymatic hydrolyses and transglycosylation reactions were analyzed by TLC on Kieselgel 60 plates (Merck, Germany) with a mobile phase of ethyl acetate/acetic acid/water (2:1:1). NMR spectra were recorded on a Bruker AMX500 operating at 500.13 MHz for ^{1}H and 125.13 MHz for ^{13}C. Chemical shifts are given relative to internal acetone, 2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C. Positive-ion mass spectra were recorded on a Micromass Q-TOF2 orthogonal acceleration quadrupole/time-of-flight mass spectrometer fitted with a nanoflow ion source (Micromass, UK) as described [20]. With the TOF analyzer operating in single-reflectron "V" mode, typical peak resolution on single-charged sodiated carbohydrate adducts was ca. 11000 FWHM. External TOF MS mass calibration was obtained over the *m/z* range 130–1980 using a solution of NaI (2 g/l) and CsI (0.05 g/l) in 1:1 PriOH/water. For accurate mass determinations in TOF MS mode, centroid spectra were generated from continuum spectra using the $(M + Na)^+$ adduct of raffinose as an internal standard (calculated mass 527.1588 Da). Centroid MS/MS spectra were produced from continuum spectra using the calculated *m/z* of the monosodiated adduct of the PNP glucooligosaccharide analyte as a reference mass.

Enzymes and substrates

Oerskovia sp. endo-β-1,3-glucanase was purified to apparent electrophoretic homogeneity as described [20]. The *Rhodothermus marinus* M133A, M133C, and M133 W mutants were obtained according to [8] and all wild type and mutant enzymes were purified to electrophoretic homogeneity as described earlier [20].

Laminarin from *Laminaria digitata*, barley 1,3-1,4-β-glucan, lichenan from *Cetraria islandica* were purchased from Sigma Chemical Co., USA. Curdlan from *Alcaligenes faecalis* was kindly donated by Prof. I.J. Goldstein, University of Michigan, USA. *p*-Nitrophenyl β-D-glucoside (PNPG) and 4-methylumbelliferyl β-D-glucopyranoside (MUG) were synthesized as described [20].

p-Nitrophenyl β-1,3-D-glucooligosaccharides ($PNPG_n$) of d.p 2–6 were synthesized from laminarin using the transglycosylating ability of *Oerskovia* endo-β-1,3-glucanase [20]. A mixture containing 600 mg of laminarin, 180 mg of PNPG and 20–30 U of the endo-β-1,3-glucanase from *Oerskovia* was incubated in 20 mM sodium acetate buffer, pH 4.8, (15 ml) at 37°C for 48 h. The extent of the reaction was tracked by TLC. Products of the transglycosylation reaction were fractionated on a Bio-Gel P2 extra fine (Bio Rad, USA) column (10 × 1200 mm, flow rate 9 ml/h) in H_2O. $PNPG_{2-4}$ were purified on an INERTSIL PREP-ODS column (20 × 250 mm) using linear gradient (0–100%) of MeCN in water. PNP laminaripentaose and PNP laminarihexaose were separated on a WATERS Carbohydrate analysis column (3.9 × 300 mm) using isocratic elution in 80% MeCN in water. Yields of $PNPG_2$, $PNPG_3$, $PNPG_4$, $PNPG_5$ and $PNPG_6$ were 30%, 22%, 18%, 9%, and 6%, respectively.

p-Nitrophenyl β-laminaribioside. MS $(M + Na)^+$ calc'd 486.1224, obs. 486.1138; NMR 1H δ: 5.276 (1H, d, $J_{H1,H2}$ = 7.63 Hz, H1), 4.779 (1H, d, $J_{H1',H2'}$ = 7.98 Hz, H1′), 3.953 (1H, d, $J_{H6a',H6b'}$ = 12.4 Hz, $J_{H6a,H5}$ = 2.3 Hz, H6a), 3.946 (1H, dd, $J_{H6a',H6b'}$ = 12.3 Hz, $J_{H6a',H5'}$ = 2.3 Hz, H6a′), 3.889 (1H, dd, $J_{H3,H4}$ = 8.7 Hz, $J_{H3,H2}$ = 9.3 Hz, H3), 3.832 (1H, dd, H2), 3.788 (1H, dd, $J_{H6b,H5}$ = 5.4 Hz, H6b), 3.719 (1H, ddd, $J_{H5,H4}$ = 9.8 Hz, H5), 3.747 (1H, dd, $J_{H6b',H5'}$ = 6.0 Hz, H6b′), 3.640 (1H, dd, H4), 3.562 (1H, dd, $J_{H3',H2'}$ = 9.3 Hz, $J_{H3',H4'}$ = 8.9 Hz, H3′), 3.517 (1 H, ddd, $J_{H5',H4'}$ = 9.8 Hz, H5′), 3.446 (1H, dd, H4′), 3.414 (1H, dd, H2′).

p-Nitrophenyl β-laminaritrioside. MS $(M + Na)^+$ calc'd 648.1752, obs. 648.1707; NMR 1H δ: 5.275 (1H, d, $J_{H1,H2}$ = 7.78 Hz, H1), 4.815 (1H, d, $J_{H1',H2'}$ = 7.98 Hz, H1′), 4.736 (1H, d, $J_{H1'',H2''}$ = 7.93 Hz, H1′′), 3.936 (1H, dd, $J_{H6a,H6b}$ = 12.4 Hz, $J_{H6a,H5}$ = 2.2 Hz, H6a),3.922 (1H, dd, $J_{H6a',H6b'}$ = 11.9 Hz, $J_{H6a',H5'}$ = 2.2 Hz, H6a′), 3.903 (1H, dd, $J_{H6a'',H6b''}$ = 12.4 Hz, $J_{H6a'',H5''}$ = 2.5 Hz, H6a′′), 3.887 (1H, dd, $J_{H3,H2}$ = 9.3 Hz, $J_{H3,H4}$ = 8.8 Hz, H3), 3.822 (1H, dd, H2), 3.789 (1H, dd, $J_{H3',H2'}$ = 9.3 Hz, $J_{H3',H4'}$ = 8.8 Hz, H3′), 3.770 (1H, dd, $J_{H6b,H5}$ = 5.4 Hz, H6b), 3.737 (1H, dd, $J_{H5',H6b'}$ = 5.4 Hz, H6b′), 3.710 (1H, ddd, $J_{H5,H4}$ = 9.8 Hz, H5), 3.707 (1H, dd, $J_{H6b'',H5''}$ = 6.1 Hz, H6b′′), 3.620 (1H, dd, H4), 3.568(1H, dd, H2′), 3.528 (1H, dd, $J_{H5',H4'}$ = 9.9 Hz, H4′), 3.516 (1H, dd, $J_{H3'',H4''}$ = 8.9 Hz, $J_{H3'',H2''}$ = 9.4 Hz, H3′′), 3.504(1H, ddd, H5′), 3.471 (1H, ddd, $J_{H5'',H4''}$ = 9.8 Hz, H5"), 3.399 (1H, dd, H4′′), 3.353 (1H, dd, H2′′).

p-Nitrophenyl β-laminaritetraoside. MS $(M + Na)^+$ calc'd 810.2280, obs. 810.2261; NMR ^{1}H δ: 5.276 (1H, d, $J_{H1,H2}$ = 7.78 Hz, H1), 4.812 (1H, d, $J_{H1',H2'}$ = 7.93 Hz, H1′), 4.779 (1H, d, $J_{H1'',H2''}$ = 7.93 Hz, H1′′), 4.730 (1H, d, $J_{H1\sim,H2\sim}$ = 7.93 Hz, H1∼), 3.934 (1H, dd, $J_{H6a,H6b}$ = 12.3 Hz, $J_{H6a,H5}$ = 2.06 Hz, H6a), 3.920 (1H, dd, $J_{H6a',H6b'}$ = 12.3 Hz, $J_{H6A',H5'}$ = 1.9 Hz, H6a′), 3.919 (1H, dd, $J_{H6a'',H6b''}$ = 11.9 Hz,

$J_{H6a'',H5''} = 2.3$ Hz, H6a′), 3.995 (1H, dd, $J_{H6a\sim H6b\sim} = 12.2$ Hz, $J_{H6a\sim,H5\sim} = 2.4$ Hz, H6a∼), 3.885 (1H, dd, $J_{H3,H4} = 8.8$ Hz, $J_{H3,H2} = 9.3$ Hz, H3), 3.8423 (1H, dd, H2), 3.779 (1H, dd, $J_{H3',H4'} = 8.9$ Hz, $J_{H3',H2'} = 9.3$ Hz, H3′), 3.768 (1H, dd, $J_{H6b,H5} = 5.4$ Hz, H6b), 3.762 (1H, dd, $J_{H3'',H4''} = 8.8$ Hz, $J_{H3'',H2''} = 9.3$ Hz, H3″), 3.747 (2H, dd, H6b′,H6b″), 3.709 (1H, ddd, $J_{H5',H4'} = 9.9$ Hz, H5), 3.703 (1H, dd, $J_{H6b\sim,H6\sim} = 5.9$ Hz, H6b∼), 3.619 (1H, dd, H4), 3.568 (1H, dd, H2′),3.546 (1 H, dd, H2″), 3.515 (1H, ddd, $J_{H5,H4} = 9.8$ Hz, $J_{H5,H6b} = 6.1$ Hz, H5′), 3.512 (1H, dd, $J_{H3\sim,H2\sim} = 9.3$ Hz, $J_{H3\sim,H4\sim} = 8.9$ Hz, H3∼), 3.511 (2H, dd, H4′,H4″), 3.500 (1H, ddd, $J_{H5'',H4''} = 9.8$ Hz, $J_{H5'',H6b''} = 6.1$ Hz, H5″), 3.468 (1 H, ddd, $J_{H5\sim,H4\sim} = 9.9$ Hz, H5∼), 3.395 (1H, dd, H4∼), 3.346 (1H, dd, H2∼).

p-Nitrophenyl β-laminaripentaoside. MS $(M + Na)^+$ calc'd 972.2808, obs. 972.2902.

p-Nitrophenyl β-laminarihexaoside. MS $(M + Na)^+$ calc'd 1134.3337, obs. 1134.3386.

p-Nitrophenyl β-laminariheptaoside. MS $(M + Na)^+$ calc'd 1296.3865, obs. 1296.4016.

p-Nitrophenyl β-laminarioctaoside. MS $(M + Na)^+$ calc'd 1458.4393, obs. 1458.4587.

MU β-laminaribioside and MU β-laminaritrioside were synthesized as described [20]. Purity of all PNP- and MU-modified β-laminarin oligosaccharides was not less than 95% according to the data obtained by ^{1}H NMR, HPLC and MS analyses. Laminaribiose and laminaritriose were produced by enzymatic digestion of laminarin by *Oerskovia* endo-β-1,3-glucanase (20 mM sodium acetate buffer, pH 4.8) followed by purification on a Bio-Gel P2 extra fine column and Lichrosorb-NH_2 column (20 × 250 mm). The β-laminarin oligosaccharides with a degree of polymerization from 4 to 6 were prepared by formic acid hydrolysis of curdlan followed by purification of individual β-laminarin oligosaccharides [25]. Purity of all β-laminarin oligosaccharides was not less than 95% estimated by ^{1}H and ^{13}C NMR analysis [26,27].

Hydrolytic activity assays

Enzyme activity in the hydrolysis of polymeric substrates was measured according to Somogyi-Nelson method [28]. Standard assays (0.1 ml) were performed in 50 mM sodium acetate buffer, pH 4.5, and contained 1 mg of laminarin and purified enzyme extract corresponding to 4–11 μg (0.009–0.01 U) of pure laminarinase. One unit of the enzyme activity produced 1 μmole of reducing sugar per min at pH 4.5, 75°C, with laminarin as a substrate. Measurement of the activity of laminarinase toward PNP and MU-containing β-laminarin oligosaccharides was performed in 50 mM sodium acetate buffer (pH 4.5) at 37°C to minimize non-enzymatic hydrolysis of the substrate. $PNPG_2$ and $PNPG_3$ hydrolysis was followed spectrophotometrically at 410 nm by measuring released PNP as described previously [29]. Enzymatic activity of the laminarinase toward MU-laminaribioside and MU-laminaritrioside was measured according to procedures reported earlier [20,30]. Analysis of PNP-containing products was performed on a WATERS Spherisorb C8 column (250 × 4.6 mm, Supelco Inc.) using a linear gradient (0–90%) of MeCN in water with spectrophotometric detection at 254 nm. Yields were estimated by integration of the corresponding chromatographic peaks.

The pH optimum for the hydrolysis of PNP-laminarioligosides was determined over the range pH 3.5–pH 8 in 100 mM citrate-phosphate buffers at 37°C using 2 mM substrate concentration.

The modes of action of the wild-type and mutant laminarinases on laminarin were studied in 50 mM sodium acetate buffer, pH 4.5, at 75°C. Typically, reaction mixtures contained 25 mg/ml of laminarin and 0.17 U of the enzyme. Aliquots were taken after appropriate time intervals, loaded onto a Dextro-Pak^{TM} cartridge column (8 × 100 mm; Millipore-Waters, USA) and fractionated using isocratic elution with water and refractometric detection.

The Michaelis-Menten parameters K_m and k_{cat} were determined from the Lineweaver-Burk plots obtained by measuring the initial rate of substrate hydrolysis. The Michaelis constant was determined for each substrate from the Michaelis-Menten equation by using nonlinear least-squares method with the ORIGIN 6.0 program. Rates were calculated for at least 8 substrate concentrations in range (0.1–6) × the K_m value.

Transglycosylating activity

^{1}H NMR studies of the laminarinase transglycosylating activities with laminarin as a substrate were carried out at a substrate concentration of 25 mg/ml in 30 mM phosphate-citrate buffer, pH 4.5, using 1.0 U of the enzyme. The reactions were carried out at 65°C for ca. 90 min. Data were acquired after a 60° pulse into 16 K points, with a spectral width of 4.6 kHz and 64 scans, including first 4 dummy scans. Total acquisition time for each spectrum was 2.06 min. Spectra were recorded at 2.1 minute-time intervals during the reaction. To analyze the products of transglycosylating and hydrolytic reactions, ^{1}H chemical shifts reported by Petersen et al. [26] were used.

Transglycosylating activities of laminarinases in the reaction of substrate transglycosylation using $PNPG_2$ and $PNPG_3$ as substrates were investigated using approx. 0.05 U of the enzyme in 20 mM sodium acetate buffer, pH 5.0, at substrate concentration of 10–30 mM, at 37°C. The course of the reaction was followed by TLC. Transglycosylation products not containing PNP were analyzed on a Dextro-Pak^{TM} cartridge column as described above. Product amounts were calculated by integration of the corresponding chromatographic

peaks; structures of β-D-glucooligosaccharides produced in the reaction of transglycosylation were verified by ^{1}H NMR analysis [26]. Yields of transglycosylation products were calculated according to Ref. [31]. PNP-containing transfer products were analyzed on a WATERS Spherisorb column as described above.

Transglycosylation reactions of the laminarinase with $PNPG_3$ and $PNPG_4$ as substrates were analyzed by ^{1}H NMR using the same reaction conditions except temperature was 40°C. Concentrations of substrate and products of the transglycosylation reaction were determined integrating the peaks characteristic for each compound. The signal at 5.27 pm corresponds to anomeric proton in glucose residue of PNPG; the signal at 4.80 pm corresponds to H1 proton of terminal glucose unit of $PNPG_2$; the signal at 4.77 pm corresponds to the H1 proton of the terminal glucose of $PNPG_3$; the signal at 4.71 ppm corresponds to the H1 proton of the glucose residue of laminaribiose; the signal at 4.73 pm corresponds to H1 of glucosidic unit of laminaritriose. The sum of intensities of the signals corresponding to α- and β-glucose anomers at 5.22 and 4.64 ppm was used for a free glucose.

The effect of pH on transglycosylation activity was measured at 37°C in the range pH 4.5–pH 8 in 100 mM citrate-phosphate buffers using $PNPG_2$ as a substrate. Transglycosylation activity was estimated by the yield of G_3 as a major product of the reaction after product separation on a Dextra-Pak^{TM} column.

Results and discussion

Asp131 and Glu134 were previously characterized as catalytically important residues in the *Rhodothermus* laminarinase [8]. Another important structural feature of the active site of bacterial laminarinases from GH16 is the presence of an invariant and catalytically important Met133 located within the active site region. Deletion of Met133 lead to dramatically reduced hydrolytic activity. Enzymes in which Met133 was replaced by Ala, Cys, and Trp retained their hydrolytic activity towards laminarin, curdlan and β-1,3(4)-glucans [8].

Table 1 gives Michaelis-Menten parameters for the hydrolysis of *p*-nitrophenyl (PNP) and 4-methylumbelliferyl (MU) $\beta-$laminaribiosides by wild-type and M133A, M133C and M133W mutant enzymes. We have shown previously [20] that MU laminaribioside and -trioside were cleaved only at the glycone-aglycone linkage as was confirmed by HPLC of the resulted products. The same should be applied to the PNP laminaribioside. Only little changes in catalytic activity against these bioside substrates were detected with any of the mutants, despite the proximity of the site of the mutation to catalytically important residues. Therefore interactions in the -1 and -2 subsites are substantially unchanged by the three mutations of residue M133.

Our initial screening of transglycosylation activity used laminarin itself as a substrate. Laminarin is based on a β-1,3-glucan main chain but also has a small proportion (5.5%) of single-sugar β-1,6-branches [32], which give characteristic ^{1}H NMR signals. Transglycosylation with laminarin results in the appearance of 1,4-linkages not otherwise present in the native polisaccharide. Proton signals H1 for glucosylated at 4 or 6 carbons appear as duplets at δ4.57–4.49, whereas H1 for glucosylated at 3 carbon in laminarin oligosaccharides, and laminarin itself, resonates at δ4.71–4.75 (4.71 laminaribiose, 4.73 laminaritriose). Consequently, the evolution with time of the ratio of the intensity of the signals in the δ4.57–4.49 region to those in the anomeric region, characteristic of the reducing ends (δ4.68–4.64, β, δ5.25–5.23, α) gives an initial measure of the transglycosylation effectiveness of various mutant enzymes. Figure 2-a shows the concept behind the assay, and Figure 2-b the ^{1}H NMR spectra of a 40 $\pm$ 4% reacted sample of laminarin. In Figure 3 the ratio of the intensity of the δ4.57–4.49 multiplet to reducing ends is plotted as a function of time for the four forms of the enzyme. It is seen that only the M133C mutant was significantly better at transglycosylation than wt.

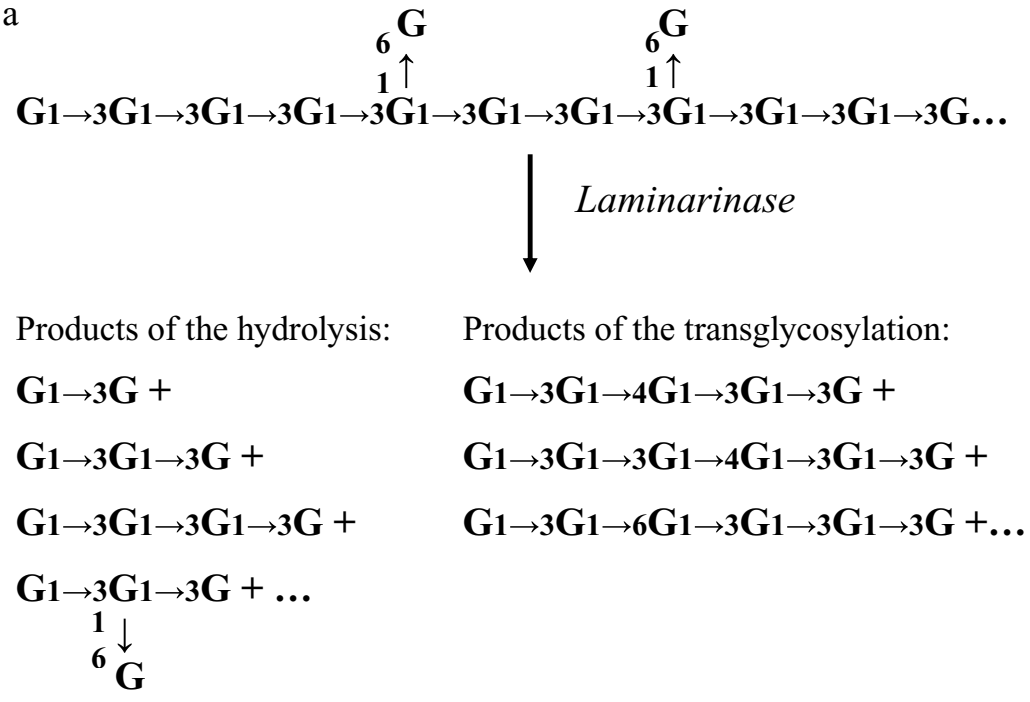
A reason for improved transglycosylation activity of the M133C mutant may be understood considering possible changes in structure of the active site of laminarinase. Three-dimensional structure of the laminarinase from *Rhodothermus marinus* is unavailable yet, but sequence similarity and known X-ray structures of enzymes from the 16 family suggest a conservative fold for all family. As a consequence, the structure of active site of β-agarase A [4] (Figure 4) which contains β-bulge and Met seems to be a good model for the laminarinase active site. Obviously, in β-agarase A, rigid structure of β-bulge is stabilized by hydrophobic interactions between Ile150 and Met151 in β-bulge and Phe137 - Phe276 located on parallel β-strands. This hydrophobic region is arranged outside of hydrophilic residues of the active site. Mutation of Met to any polar amino acid residue like Cys could cause significant reorganization in the active site, namely, in position of proton donor – Glu152 (analog of Glu134 in the laminarinase). In view of that, the second step of the double displacement mechanism reaction may be slower, resulting in a long-lived glycosyl-enzyme intermediate produced during the first step. An increasing life time of glycosyl-enzyme intermediate, accordingly, may increase the probability of transglycosylation [13,14]. The above assumption explains why M133W or M133A mutants did not reveal changes in transglycosylation activity in comparison with the wild type enzyme. Both of them have hydrophobic residues instead of hydrophobic Met that did not affect the structure of the active site. Conversely, polar Cys residue will be exposed out of the mentioned hydrophobic region disturbing geometry of the active site.

Table 1 Kinetic characteristics of a native and mutant forms of the laminarinase

	$MUFG_2$			$MUFG_3$			$PNPG_2$		
Enzyme	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $(s \cdot mM)^{-1}$	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $(s \cdot mM)^{-1}$	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $(s \cdot mM)^{-1}$
Wild type	0.1 ± 0.002	0.10 ± 0.005	1.0 ± 0.07	0.02 ± 0.001	0.13 ± 0.007	6.5 ± 0.1	0.37 ± 0.01	0.55 ± 0.06	1.4 ± 0.1
M133C	0.1 ± 0.002	0.095 ± 0.004	0.95 ± 0.06	0.018 ± 0.001	0.12 ± 0.007	6.6 ± 0.1	0.35 ± 0.01	0.53 ± 0.06	1.5 ± 0.1
M133A	0.09 ± 0.002	0.12 ± 0.005	1.2 ± 0.06	0.017 ± 0.001	0.14 ± 0.008	7.6 ± 0.2	0.33 ± 0.01	0.050 ± 0.05	1.5 ± 0.1
M133W	0.11 ± 0.002	0.12 ± 0.005	1.09 ± 0.06	0.019 ± 0.001	0.12 ± 0.008	6.3 ± 0.2	0.37 ± 0.01	0.052 ± 0.05	1.4 ± 0.1

Measurement of the activity of laminarinase toward PNP and MU-containing β-laminarin oligosaccharides was performed in 50 mM sodium acetate buffer (pH 4.5) at 37°C. The values are an average of at least 3 determinations.

a



b

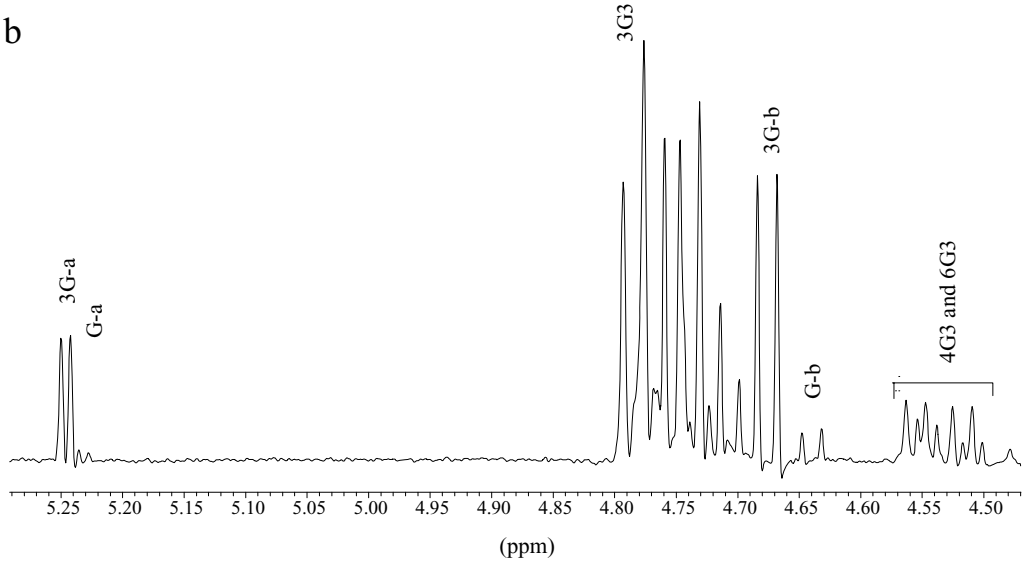


Fig. 2 **a:** Schematic presentation of the products' distribution in the reaction of the substrate transglycosylation. **b:** ^{1}H NMR spectrum of the laminarin degradation effected by the laminarinase: G-a and G-b, proton signals corresponding to free α- and β-glucose, respectively; 3G-a and 3G-b, α- and β-1,3-glucosidic bonds reducing terminals; 3G3, 4G3, and 6G3, signals H1 for glucosylated at 3,4, and 6 carbon, respectively

Michaelis-Menten parameters determined for the hydrolysis of β-laminarioligosaccharides of dp 2–6 are displayed in Table 2. After about 15% conversion, the resulting products were identified with HPLC. In all cases, holosidic cleavage was a minor pathway, accounting for at most 22% of the total reaction. Michaelis-Menten parameters refer to the total reaction, not heterosidic cleavage.

There were no detectable differences between the action patterns of the mutant and the wt (Figure 5). In case of $PNPG_5$ and $PNPG_6$ similar numbers of the products are formed, which confirms the conclusion from HPLC analysis that production of extended β-glucooligosaccharides with d.p. 4–6 by M133C mutation is a result of improved transglycosylating activity and not caused by changes of mode of action. The predominance of heterosidic cleavage in the hydrolysis of aryl oligosaccharide glycosides by endoglycanases is unusual. In the present case the ability of the +1 subsite to accommodate a nitrophenyl group may

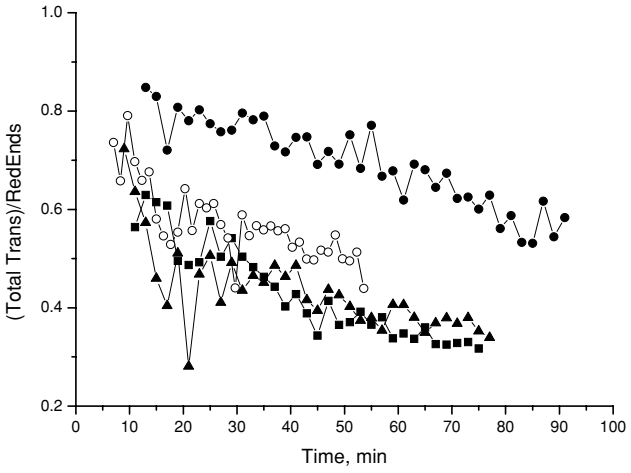


Fig. 3 Time-dependence of the formation of transglycosylation products expressed by the ratio of reducing ends against transfer product yields in the hydrolysis of laminarin of wild-type and mutant enzymes: ◯, wild-type; ●, M133C; ■, M133W; ▲, M133A.

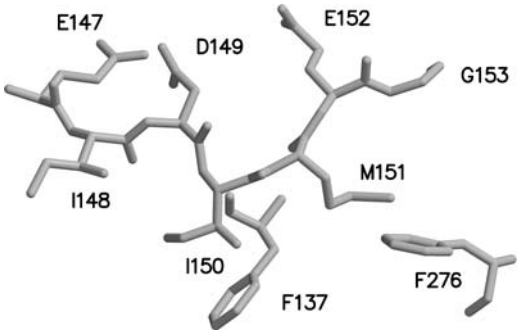


Fig. 4 The structure of the active site of the β-agarase A (PDB code 1O4Y) used as a model for the *Rhodotermus* laminarinase active site. The image was prepared using program MOLSCRIPT [33].

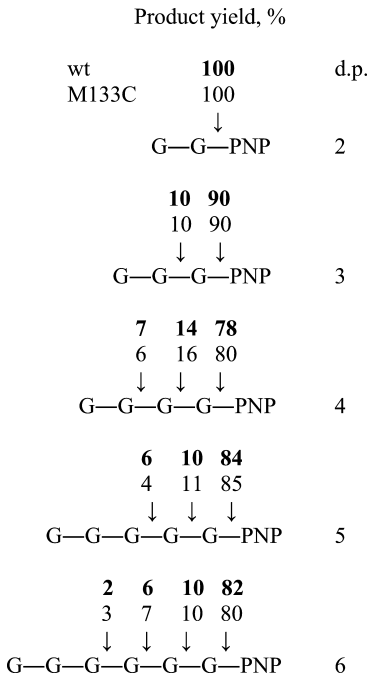


Fig. 5 Action patterns of wild-type and M133C β-1,3(4)-glucanases with PNP laminarioligosaccharides (d.p. 2–6). G, glucosyl residues; PNP, *p*-nitrophenyl groups; d.p., degree of polymerisation. Numbers above the arrows represent percentages of cleavage frequencies of glycosidic bonds. Bold numbers are for wild-type enzyme, normal numbers–for mutant M133C. The values are an average of at least 3 determinations.

be connected with the very wide range of leaving group specificities in Family 16 [2].

The effect of each successive glucose residue on k_{cat}/K_m (for hydrolysis with heterosidic cleavage only) was used to calculate subsite affinities for subsites −3, −4, −5 and −6. The results are given in Table 3. It is seen that subsites −5 and −6, whilst measurably increasing catalytic efficiency, appear to be of minor importance. Absolute values of k_{cat}/K_m (10^6 M^{-1} s^{-1} for the longer substrates), though, may be close to the diffusion limit for the binding of a large substrate to the cleft of an endoglucanase in a defined way.

A critical assumption of subsite analysis is that the affinity of one subsite is independent of the occupancy of the others; this has been shown to be incorrect for some enzymes [34,35].

The pH optima for all substrates with both enzymes were a common pH 4.5 (data not shown). As there was little or no difference between wt and mutant in the affinities of the

Table 2 Kinetic parameters of PNP β-laminarioligosaccharide hydrolysis by the wild-type and M133C laminarinases

	K_m, mM		k_{cat}, s^{-1}		k_{cat}/K_m, $(s \cdot mM)^{-1}$	
Substrate	Wild-type	M133C	Wild-type	M133C	Wild-type	M133C
$PNPG_2$	0.37 ± 0.01	0.35 ± 0.01	0.55 ± 0.06	0.53 ± 0.06	1.4 ± 0.1	1.5 ± 0.1
$PNPG_3$	0.014 ± 0.001	0.014 ± 0.001	1.2 ± 0.1	1.1 ± 0.1	85 ± 3	78.5 ± 3
$PNPG_4$	0.012 ± 0.001	0.015 ± 0.001	1.9 ± 0.1	1.85 ± 0.1	158 ± 12	168 ± 12
$PNPG_5$	0.010 ± 0.002	0.010 ± 0.002	2.05 ± 0.1	1.9 ± 0.1	205 ± 15	190 ± 15
$PNPG_6$	0.0085 ± 0.0002	0.0081 ± 0.0002	2.0 ± 0.1	1.9 ± 0.1	238 ± 18	240 ± 18

Standard assays were performed in 50 mM sodium acetate buffer, pH 4.5, 37°C as described in Materials and methods. The values are an average of at least 3 determinations.

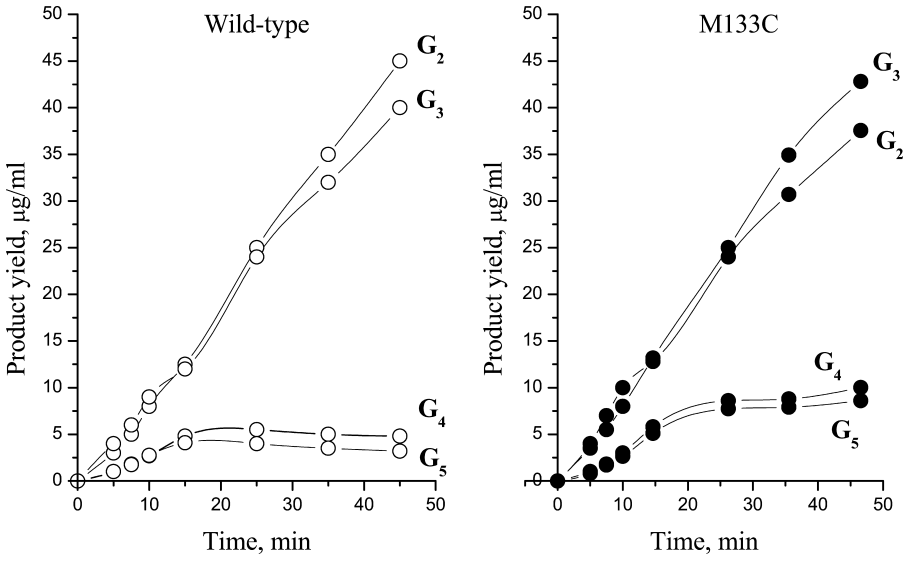


Fig. 6 Kinetics of product formation during the hydrolysis of laminarin of *L. digitata* catalysed by wild type laminarinase (A) and M133C (B). G_2, β-glucobioside; G_3, β-glucotrioside; G_4, β-glucotetraoside; G_5, β-glucopentaoside. The values given are an average of at least 3 determinations.

Table 3 Contribution of subsites to transition-state stabilization

	$\Delta G^*_{subsite}$, kcal/mol	
Subsite	Wild type	M133C
−III	−2.5	−2.4
−IV	−0.39	−0.45
−V	−0.15	−0.09
−VI	−0.09	−0.14

negative subsites, a higher concentration of longer oligosaccharides during hydrolysis of laminarin can be taken as an indication of increased transglycosylating activity. The time courses of the appearance of laminarioligosaccharides from laminarin, catalysed by wt and M133C mutant are shown in Figure 6; the tetrasaccharide and pentasaccharide (and a trace of hexasaccharide) are produced in greater amount by the mutant, and are more persistent.

The transglycosylation reaction was examined with wt laminarinase with both PNP laminaribioside and –trioside, at concentrations between 10 and 30 mM. At an initial PNP laminaribioside concentration of 30 mM, 20 ± 3% laminaritriose and 10 ± 3% laminaritetraose were produced, in addition to hydrolysis products. Trace amounts of their p-nitrophenyl glycosides were also detected. With PNP laminaritrioside (30 mM) as substrate, yields of laminaritetraose and -pentaose were 20% and 15%, respectively, with, again, trace amounts of their *p*-nitrophenyl glycosides. Figure 7 displays the TOF mass spectra of these reaction mixtures. The absence of large quantities of PNP-laminarioligosaccharide glycosides, formed by transglycosylation, is readily understood, since, once formed, they would be preferentially hydrolysed at the heterosidic linkage.

To evaluate the efficiency of transglycosylation for both wt and M133C mutant, the ratio between quantities of transglycosylation acts and the sum of possible catalytic acts (hydrolysis and transglycosylation) was used:

$$k = \frac{N_{\text{trans acts}}}{N_{\text{hydrol acts}} + N_{\text{trans acts}}}, \quad (1)$$

where $N_{\text{trans acts}}$ was evaluated by ^{1}H NMR analysis based on concentration of transfer products G_n, $n > 3$ (for $PNPG_3$) and $n > 2$ (for $PNPG_2$). $N_{\text{hydrol acts}}$ was assumed to be equal the quantity of reducing ends calculated as a sum of glucose, glucobiose units and G_n ($n > 2$). Thus, for the M133C mutant and wt, the values of k were 0.29 and 0.24 when $PNPG_3$ was a substrate and 0.27 and 0.20 in case of $PNPG_4$.

The stereochemistry and regiochemistry of the transfer products were confirmed by ^{1}H NMR, the spectra of oligosaccharides corresponding to those reported by Petersen *et al*. for laminarin oligosaccharides [26]. The absence of detectable quantities of 1,4-linked transfer products from low MW substrates is puzzling, in the light of the results for laminarin itself. Possibly occupancy of remote positive subsites (+4, +5) can alter the orientation of the sugar ring in the +1 subsite.

Conclusion

In the present work, we analyzed the effect of substitution of the non-catalytic acidic residue Met133 located within the active site region on hydrolytic and transglycosylating activities of the laminarinase of *Rhodothermus marinus*. Among the mutants, M133A, M133W and M133C, only M133C, was found to have improved transglycosylation activity

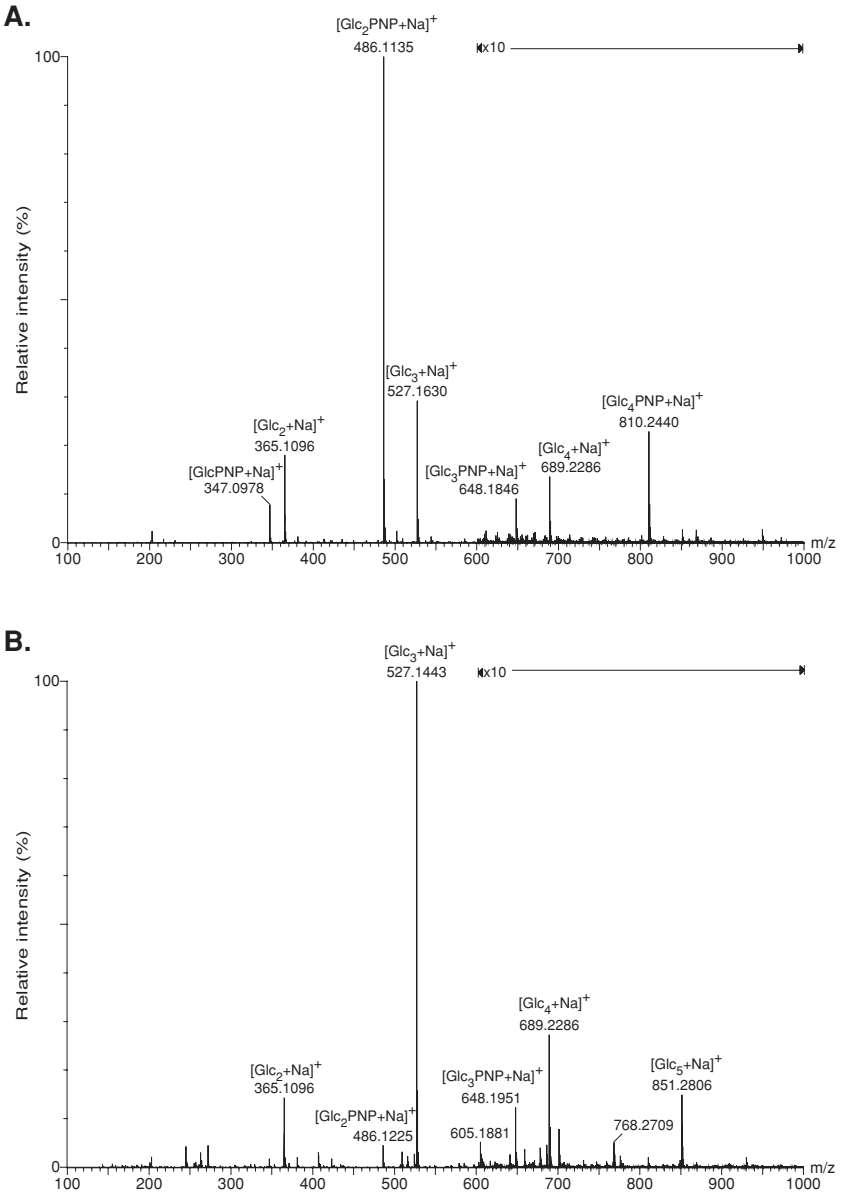


Fig. 7 Mass spectra of $PNPG_2$ (A) and $PNPG_3$ (B) hydrolysis catalysed by the laminarinase.

indicated by increase of extended oligosaccharides during the reaction with β-1,3- and β-1,3-1,4-homo- and heteroglucans. Replacement of Met133 did not affect regiospecificity during transglycosylation. Comparison of hydrolytic properties of all mutant forms of the laminarinase in the hydrolysis of a number of synthetic substrates showed only slight differences of the hydrolytic characteristics, k_{cat} and K_M. It was found that wild-type enzyme and its mutant M133C, with increased transglycosylating activity, exhibited similar patterns of action indicating that substitution of amino acid residue in the catalytic pocket of the enzyme causes changes of transglycosylating activity and did not influence substrate affinities within the active center of the laminarinase.

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