The laminarinase from thermophilic eubacterium *Rhodothermus marinus*

Conformation, stability, and identification of active site carboxylic residues by site-directed mutagenesis

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A gene (*lamR*) encoding laminarinase (*LamR*) was cloned from the marine thermophilic eubacterium *Rhodothermus marinus* ITI278. The enzyme purified from recombinant *Escherichia coli* cells hydrolyses mixed 1,3-,1,4-,β-glucans (lichenan, barley and oat β-glucan) and 1,3-,β-homoglucans (laminarin, curdlan) by an endo type action pattern. The CD spectrum of laminarinase is characteristic for a protein with prevailing β secondary-structural elements, and the fluorescence spectrum points to a surface localisation of the tryptophan residues. A half-transition concentration of 5.4 M guanidinium chloride was measured for the denaturant-induced unfolding of laminarinase monitoring changes of the ellipticity at 222 nm and the fluorescence. Substitution of acidic residues Glu34, Asp29, Asp33 by Ala, Cys or Trp did not affect activity against 1,3-,1,4-,β-polyglucans and 1,3-,β-polyglucans, but in mutant Met133A the rate of hydrolysis of cellobiosyltriose (G14G13Gr) was increased about 10 times. Hydrolysis of 1,3-,β-oligosaccharides and 1,4-,β-oligosaccharides (DP 2-7) demonstrated the ability of the enzyme to cleave 1,3-,β-linkages and 1,4-,β-linkages in low-molecular-mass carbohydrates independent of the structure of neighbouring linkages. The laminarinase contains five or six subsites for substrate binding according to the action pattern deduced from hydrolysis of labelled and unlabelled curdlan oligosaccharides of different chain length.

**Keywords:** endo-1,3-,β-glucanase; conformation; stability; laminarinase; *Rhodothermus*; active-site residue.

Mixed linkage β-glucans such as cereal glucan and lichenan containing 1,3-,β-linkages and 1,4-,β-linkages are attacked by three types of endo-β-glucanases: endo-1,4-,β-glucanases, lichenases, and endo-1,3-,β-glucanases. The enzymes are distinguished by their specificities and substrate spectra [1]. Endo-1,4-,β-glucanases, or cellulases, hydrolyse substrates containing adjacent glucopyranosyl residues that occur in homo 1,4-,β-glucans as carboxymethyl cellulose and mixed linked β-glucans in which two or three adjacent 1,4-,β-linked glucose units are combined via a single 1,3-,β-linkage. These enzymes exhibit no similarity in their primary structure to representatives of the two other groups of 1,3-,1,4-,β-glucan hydrolyases. Lichenases and endo-1,3-,β-glucanases (laminarinases) are more related in their primary structures and both have been classified as members of family 16 of glycosyl hydrolases [2, 3]. They are clearly different from other bacterial and plant laminarinases which cleave exclusively 1,3-,β-linkages adjacent to another 1,3-,β-linkage(s) and are not able to cut 1,4-,β-linkages also if neighboured to a 1,3-,β-linkage in mixed linked β-glucans. Representatives of that group have been classified into glycosyl hydrolase family 17 and do not share any sequence similarities with the laminarinases belonging to glycosyl hydrolase family 16 [2, 3].

Lichenase 1 and 1,3-,1,4-,β-D-glucan 4-glucanohydrolase was carefully characterised as a retaining glycosidase with extraordinary substrate specificity exclusively hydrolysing 1,4-,β-linkages, where the glucosyl residue is itself linked at the (O)3 position [4–6]. Within the highly conserved sequence WDEIDIE in *Bacillus macerans* lichenase Glu103 and Glu107 were identified to represent the catalytic nucleophile (Glu103) and the general acid (Glu107) responsible for cleavage of the 1,4-,β-glycosidic...
bond [7, 8]. Endo-1,3-(1,3,1,4)-β-D-glucan 3(4)-glucanohydro-lase or laminarinase is less specific and able to hydrolyse 1,3-β-homoglucans (laminarin, curdlan), artificial pneumococcus poly-glucanase as one of the structural ele-

ments determining substrate specificity of laminarinases was demonstrated by deletion and by substitution with other resi-
dues.

MATERIALS AND METHODS

Organisms and growth conditions. R. marinus ITI 278 ob-
tained from the strain collection of the Technological Institute of Iceland was grown at 65°C in medium 162 supplemented with 2% NaCl [12]. Excherichia coli strains DH5a and BL21 (DE3) harbouring plasmid pLysE [16] were used as host strains for the recombinant plasmids pMK1, pMKM1 and pMMKlex, respectively. E. coli strains were grown in Luria-Bertani medium supplemented, if necessary, with ampicillin (final concentration: 100 μg/ml). Isopropylthio-
D-glactoside was added at a final concentration of 0.4 mM for the induction of gene expression.

Enzymes and substrates. Restriction enzymes and DNA-
modifying enzymes were purchased from Appligene. Laminarin from Laminaria digitata, lichenan from Cetraria islandica, curdlan from Alcaligenes faecalis, and cello-oligosaccharides were obtained from Sigma, oat β-glucan was obtained from Megazyme.

Cloning of the lamR gene. Chromosomal DNA was pre-
pared from 35 ml R. marinus overnight culture and further puri-
ified by CsCl density gradient centrifugation. Based on the bgI sequence of R. marinus strain ITI378, primers were designed to amplify the lamR gene by the PCR on a DNA Thermal Cycler (MWG-Biotech). The two primers were as follows: RM1, 5'-CCTGTCTTCTAGACAACCCACCGGC, sense; and RM2, 5'-AGAGCTTATGGCGGCCTAGCAACAGC, antisense. Restriction sites for XbaI (RM1) and SpHl (RM2) are underlined. In addition to the template (0.1 μg) and the primers (25 ng), the 50-μl reaction mixture contained 0.2 mM dNTPs, Taq DNA polymerase buffer, 5 mM MgCl2, and 2.5 U Taq DNA polymerase (Repli-
therm, Biozym) and 1 U Deep Vent DNA polymerase (New England Biolabs) and was subjected to 25 cycles of amplifica-
tion (30 s at 94°C, 30 s at 55°C, and 45 s at 72°C). A PCR product with the expected size was digested with XbaI and SpHl, ligated into pTZ18 vector plasmid and used for transformation into DH5α cells. The yielding recombinant plasmid pMK1 was sequenced by the dideoxynucleotide chain-termination reaction [17] with fluorescence labelled primers either using the Auto-
Read Sequencing Kit (Pharmacia) or the Cycle Sequencing Kit

Fig. 1. Sequence comparison of the active-site region of B. macerans β-glucanase with other members of glycosyl hydrodase family 16 and Trichoderma enzyme CBH1. Sequences within the active site area of B. macerans and B. licheniformis β-glucanases are identical. Note that also representatives of eukaryotic organisms belong to family 16. Residues identified as catalytic amino acids in B. macerans β-glucanase and CBH1 are labelled by vertical arrows. Abbreviations: lic, lichenase or 1,3-β-glucanase, R.m., R. marinus

Alcaligenes faecalis

Anopheles gambiae

Arabidopsis thaliana

Clostridium thermocellum

Rhizopus arrhizus

Thermotoga neapolitana

Ox., Oerskovia saxeheinizofytica (Cellulosomus cellulosan);
B.c., Bacillus circulans;
B. licheniformis; B. licheniformis exoK

B. licheniformis

Rhizobium melilotii

Bacillus subtilis C.; Clostridium thermocellum F.; Fibrobacter succinogenes; ExoK R.m. exoK gene product from Rhizobium melilotii; Meri A.th.; meril gene product from Arabidopsis thaliana; XET T.m., xylolucan endo transglycosylase from Tropea-Diella maya; lam, laminarinase or 1,3-β-glucanase, R.m., R. marinus (within the active-site region sequences of strains ITI378 and ITI78 are identical); T.n., Thermotoga neapolitana; O.x., Oerskovia saxeheinizofytica (Cellulosomus cellulosan); B.c., Bacillus circulans; S.p., Stronglyocentrotus purpuratus; CFG crab, coagulation factor G from Tachypleus tridentatus, BBP A.g., putative gram-negative-bacteria-binding protein from Anopheles gambiae.
The region coding for LamR without the signal-peptide code was amplified from plasmid pMK1 with the primer pair RM4 (sense), 1224 (New England Biolabs, antisense) by PCR. The sense primer generating an EcoRV restriction site (underlined) and a ribosome-binding site at the 5′ end was as follows: RM4, 5′-GAAGATATATAAGAGAATATATGAGCCGGCAGCACGCG-3′.

The amplified 931-bp fragment was digested with EcoRI and HindIII, and ligated into pTZ18 vector plasmid and transformed into DH5α cells. The resulting recombinant plasmid pMK1 harbouring the lamR gene coding region without the signal peptide was used to transform DH5α cells.

Mutagenesis of the lamR gene. Base substitutions were introduced by combining the splicing by overlapping extension method (SOE, [18]) and the cassette mutagenesis variant [19] of PCR. At first, the primers RM6 (sense) and RM5 (antisense) were used to introduce a silent mutation generating an EcoRV restriction site into the active-site region of lamR at bp 666 (bp numbering here and in the following refers to numbering in sequence AF047003). RM6, 5′-AGATCTGAT-ATCATGAGGCCAG-3′, and RM5, 5′-TCCATGAT-ATCATGCTGCC-3′. Base substitutions are labelled by asterisks; EcoRV restriction sites are underlined.

Afterwards, an amplified sequence containing the desired mutation was introdced by replacing a DNA fragment of the lamR site gene either upstream or downstream from the created EcoRV site. Plasmid pMK1 was used as template DNA for mutagenesis and as cloning vector. Primers used for mutagenesis are listed below: E4, sense, 5′-CGAGATCATGACATGACG-3′; M1, antisense, 5′-CGCTCCATGATCATGCTGCC-3′ (mismatch at bp 670); M2, antisense, 5′-GCTCCATGATCATGCTGCC-3′ (mismatch at bp 658); E3, 5′-GGATCTGATGATCCAGC-3′ (mismatch at bp 675); E2, 5′-TCCATGATCGCTGCAC-3′ (mismatch at bp 671). The mutations were confirmed by sequence analysis.

Expression, purification and analysis of recombinant laminarinase LamR from R. marinus. The primer Rmex1 was designed to control the expression of the mature lamR gene product by the T7 polymerase system. Rmex1, 5′-ATATAGCATGGACGGCGCGCGACGC-3′, the Ncol restriction site is underlined. The DNA fragment amplified from pMK1 with Rmex1 and 1224 was digested with Ncol and BamHI, and cloned into pRSETd vector [16], resulting in pMKM1ex. Recombinant LamR wt and mutant proteins were expressed in E. coli BL21 ployS cells after isopropylthio-ß-D-galactoside induction. Cells were sonicated for 10 min in 10 mM Tris, pH 8.0, containing 5 mM EDTA and 0.02% NaN3. Cell debris were removed by centrifugation (10000 g) for 10 min. The resulting supernatant was adjusted to pH 5.5 and heated at 60°C for 20 min. The precipitated proteins were removed by another centrifugation. The supernatant was dialysed against 20 mM sodium acetate, pH 5.0, 5 mM CaCl2, and loaded on an S-Sepharose Fast Flow column (Pharmacia) equilibrated in 20 mM sodium acetate, pH 4.48, 5 mM CaCl2. Bound proteins were eluted with a linear gradient of NaCl (0 to 1 M) with a flow rate of 1 ml/min. Fractions containing the glucanase were pooled and desalted on a gel-filtration column Superdex 75 HR10/30 (Pharmacia). The protein was stored at 4°C in 50 mM sodium acetate, pH 5.5, 2 mM CaCl2, and 0.02% NaN3.

Extracellular, periplasmic and cellular fractions were isolated as described by Cornelis et al. [20]. Periplasmic β-glucanase was taken as the activity found in the supernatant following osmotic shock with cold water.

Protein concentrations were determined spectrophotometrically at 282 nm [21] or by the dye-binding method according to Bradford [22]. An absorption coefficient A1%λ= 32.38 ml · mg⁻¹ · cm⁻¹ at 282 nm was calculated from the amino acid composition using the program PHYSCHEM of the program package PCGENE (Intelligenetics).

N-terminal amino acid sequence analysis was performed using a liquid-phase sequenator (Beckman Instruments, Model 890C).

CD spectra were measured with a Jasco J-720 spectropolarimeter (Jasco Instruments S.A.), and fluorescence spectra with a Shimadzu RFPC5001 fluorimeter (Shimadzu) as described previously [21]. CD spectra in the far-UV far ultraviolet were recorded at 25°C, a path length of 0.01 cm and a protein concentration of 0.5 mg/ml in 5 mM sodium acetate, pH 5.5, 0.5 mM CaCl2. Molar ellipticities are expressed as mean residue molar ellipticity [Q] (deg · cm² · dmol⁻¹) using a mean residue molecular mass of 116.3.

The content of secondary-structure elements was determined using the variable selection method (VARSCL1 program) [23]. The calculations were started with 33 reference proteins and some protein spectra were eliminated systematically until the recommended selection criteria for good evaluations were fulfilled, namely a value of 1 ± 0.01 for the sum of secondary-structure elements (α-helices, β-structures, turns and other structures), absence of negative values for secondary structure elements, and a good fit of the calculated spectrum to the experimental one (rms error < 0.17).

Fluorescence measurements were performed at an excitation wavelength of 295 nm and with 5-nm bandwidths for both the excitation and emission monochromator. Concentrations of protein were 25 µg/ml, concentrations of carbohydrates and oligosaccharides were 5 mg/ml, except 20 µg/ml of mixed-linkage trisaccharides G1-4G1-3G. Fluorescence measurements were performed at an excitation wavelength of 295 nm and with 5-nm bandwidths for both the excitation and emission monochromator. Concentrations of protein were 25 µg/ml, concentrations of carbohydrates and oligosaccharides were 5 mg/ml, except 20 µg/ml of mixed-linkage trisaccharides G1-4G1-3G.

Equilibrium unfolding transition curves of laminarinase were measured at 25°C monitoring changes of the ellipticity, θ, at 222 nm and the fluorescence intensity ratio I110 nm/I300 nm. The protein was in 20 mM sodium acetate, pH 5.5, 5 mM CaCl2, at a concentration of 0.065 mg/ml, and the path lengths were 0.1 cm for CD and 0.3 cm for fluorescence measurements. Guanidinium chloride was added to the samples to the desired concentrations. To achieve unfolding equilibrium the samples were incubated for at least 18 h at room temperature before measurements.

The fraction f0 of unfolded laminarinase was calculated according to Pace and Scholtz [24]. Thermal unfolding in buffer containing different guanidinium chloride concentrations was analysed measuring the ellipticity at 222 nm using a heating rate of 20°C/h.
Preparation, purification and labelling of oligosaccharides. Oligosaccharides were prepared from an acid hydrolysate of curdlan according to the method of Ogawa [25]. Oligosaccharides DP 1–10 were separated using Toyopearl gel-filtration resin HW-40S (TosoHaas). Two jacked columns (each 1.6 cm × 95 cm) were tandemly connected and equilibrated with water. Sugars were separated at 65°C with a flow rate of 0.5 ml/min and refractive index detected (Refractive index monitor RI132, Gilson). Following TLC analysis, fractions containing oligosaccharides of identical size were pooled, freeze-dried and rechromatographed. Gel filtration was repeated until individual oligosaccharide preparations were more than 95% pure as judged by either TLC or HPLC.

The concentration of sugars was determined using 200 μl of an oligosaccharide solution, adding 1 ml 86% sulphuric acid, with 700 mg/l l-cysteine hydrochloride, to 750 μl of water. Sugars were separated at 65°C with a flow rate of 0.5 ml/min and refractive index detected (Refractive index monitor RI132, Gilson). Following TLC analysis, fractions containing oligosaccharides of identical size were pooled, freeze-dried and rechromatographed. Gel filtration was repeated until individual oligosaccharide preparations were more than 95% pure as judged by either TLC or HPLC.

RESULTS
Cloning, sequence analysis and expression of LamR. The gene encoding the β-glucan–hydrolysing enzyme from R. marinus ITI278 was amplified from chromosomal DNA with sequence-specific primers annealing with the 5′ and 3′ region of the nucleotide sequence of the bgla gene from R. marinus ITI378 [12]. The amplified DNA fragment covering bp 74–1191 (numbering refers to the bgla sequence) was cloned into pTZ18 yielding plasmid pMK1. Recombinant E. coli cells transformed by pMK1 were shown to hydrolyse lichenan, suggesting the expression of an active β-glucan–hydrolysing enzyme. The cells were also found to hydrolyse 1,3-, 1,4-β-glucans such as laminarin and curdlan. Sequence analysis revealed 89.6% similarity with the bgla gene sequence from R. marinus ITI378. The sequence contains one ORF (lamR) encoding 276 amino acid residues. A putative ribosome-binding site ACGCAGG is located five nucleotides upstream from the first of the two ATG codons at the beginning of the ORF. Major differences to the bgla gene of ITI 378 exist near the 5′-end of lamR, namely within the 5′-region preceding the putative translation-start codon and within the sequence encoding a putative signal peptide and few N-terminal residues of the mature protein. Minor differences resulting in only 6 amino acid substitutions were detected within the region encoding the remaining 249 amino acids of the mature protein. N-terminal sequencing of the protein expressed in recombinant E. coli cells (pMK1) revealed the sequence DGDQPIRRL. This suggests a length of 257 amino acids (calculated molecular mass: 29900) for the mature protein, and the processing of an N-terminal extension of 19 amino acids (calculated molecular mass: 2128). The sequence of the N-terminal extension, MMQRFILCSLL, displays features more related to a membrane-spanning helix than to a typical prokaryotic signal peptide. About 80% of the total activity detected in recombinant E. coli cells were located within the periplasmic space. In R. marinus only minor activities were present in the periplasmic fraction whereas the main laminarinase activity was detected in the cell fraction consisting of cytoplasm and membranes. Yzymograms prepared from R. marinus cell extracts proved the existence of two β-glucan–hydrolysing isoenzymes with apparent molecular masses of 32 kDa (processed) and 34 kDa (unprocessed); whilst recombinant E. coli cells only contained the 32-kDa form (Fig. 2).

The level of expression was about five-times higher when the sequence coding for the signal peptide had been removed (pMKM1). As expected, the whole activity was present in the cytoplasm. Under control of the T7 polymerase promoter (pMKMlex) further increase of gene expression was achieved. Up to 13 mg/l culture, that is about 125-times more than produced by native R. marinus cells, were obtained from recombinant BL21pLysS cells.

CD measurements. The CD spectrum of the recombinant protein LamR is shown in Fig. 3A. The spectral characteristics point to a relatively low content of α-helical structures and to larger contributions of β-structures. For comparison, in Fig. 3A the CD spectrum of the B. macearenas endo-1,3,1,4-β-glucanase is given. From the comparison of the spectral characteristics a basically similar type of structural organisation of the two enzymes can be assumed. In detail, significant differences between the secondary structures of these two members of glycosyl hydrolase family 16 are indicated. The CD spectra were evaluated
Fluorescence measurements. Fluorescence emission spectra obtained by excitation at 295 nm are shown in Fig. 3B. Laminarinase contains 14 tryptophan residues. The spectrum is a composite property of the fluorescence of all tryptophan residues, but contributions of individual residues may vary largely. The observed peak position at 346 nm indicates a rather hydrophobic surrounding suggests a favoured surface localisation of at least those tryptophan residues which dominate the fluorescence spectrum.

Fluorescence emission spectra of LamR are reduced in their intensities and displayed a shift of maximum of 19 nm when the protein was incubated with the substrates oat glucan, laminarin or G1-4G3Gr, but were not altered in the presence of amylose or carboxymethyl cellulose. The fluorescence emission spectra of the B. macerans β-glucanase were not changed in the presence of the same carbohydrates (data not shown).

The fluorescence maximum at 327 nm of LamR indicates a hydrophobic surrounding of those tryptophans which dominate the spectrum in the presence of substrates. Obviously, the substrates cannot interact in specific manner with all 14 Trp residues of the enzyme. It is not possible to exclude substrate-induced conformational changes of the enzyme causing the observed spectral shift and reduction in emission intensity. However, more probably the fluorescence changes are caused by an efficient quenching of some of the more solvent-exposed Trp residues, so that fluorescence of the more buried ones dominates the spectrum of the complex.

Stability against guanidinium chloride and temperature. Unfolding of LamR at increasing GdnHCl concentrations and temperatures was monitored measuring changes of circular dichroism and fluorescence. While changes of the ellipticity at 222 nm and 210 nm reflect alterations in the secondary structure, changes of the fluorescence correlate with unfolding of the tertiary structure.

Fig. 4A shows the standardised transition curves of LamR and B. macerans β-glucanase. Unfolding of B. macerans β-glucanase occurs at a half-transition concentration of 2.5 M Gdn/HCl and can be described by a two-state model [29]. Unfortunately, the two-state model could not be proved for the unfolding of LamR. Thus, the determination of $AG$ values was not possible. For LamR a smooth sigmoidal unfolding curve with a half-transition concentration of about 5.5 M Gdn/HCl was found. The cooperativity of unfolding of LamR is lower than that of glucanase and the formation of unfolding intermediates cannot be excluded from such data. However, further tests to establish the mechanism of unfolding, e.g. differential scanning calorimetric analyses, could not be performed because of the irreversibility of the thermal unfolding even in buffer containing guanidinium chloride. In Gdn/HCl-free buffer at temperatures close to 100°C, strong precipitation occurred. Precipitation was reduced at 0.5 M and not evident at higher Gdn/HCl concentrations. Therefore, thermal unfolding monitoring changes of the ellipticity at 222 nm was studied in the presence of various concentrations of Gdn/HCl as shown in Fig. 4B. The $t_m$ values decrease linearly with increasing denaturant concentrations (Fig. 4B) from 88°C in 1.05 M Gdn/HCl to 54°C in 5.2 M Gdn/HCl. Extrapolation to 0 M Gdn/HCl yields a half-transition temperature of 97°C. Control measurements of the CD spectra at 25°C before and after heating of the samples clearly indicated spectral differences (data not shown) and thus, irreversibility of unfolding. Therefore a more sophisticated thermodynamic analysis was not justified.

Substrate specificity and biochemical properties of LamR. The hglA gene product from R. marinus ITI378 was previously
characterised as an enzyme hydrolysing mixed-linkage $\beta$-glucans and 1,3-$\beta$-homoglucans [12]. LamR, the product of the \textit{lamR} gene from \textit{R. marinus} ITI2778 purified from recombinant \textit{E. coli} cells, shows a comparable specificity. LamR hydrolyses mixed-linkage $\beta$-glucans (specific activity against lichenan 3111 \text{U/mg}; oat $\beta$-glucan 2199 \text{U/mg}), and 1,3-$\beta$-homoglucans such as laminarin (specific activity: 656 \text{U/mg}). LamR did not attack carboxymethyl cellulose, avicel and oat spelt xylan. This broad substrate specificity characterises the enzyme as a bacterial laminarinase, which hydrolysing 1,3-$\beta$-homoglucans and mixed-linkage $\beta$-glucans containing alternating 1,3-$\beta$-linkages and 1,4-$\beta$-linkages [1]. Hydrolysis of 1,3-1,4-$\beta$-glucan and laminarin follows the endo type action pattern. This was proved by intermediate appearance of oligosaccharides with DP > 4 detected after short incubation time (Fig. 5). Hydrolysis of 1,3-$\beta$-oligosaccharides and 1,4-$\beta$-oligosaccharides ranging from DP5
to DP2 suggests that LamR cleaves 1,3-β-linkages and 1,4-β-linkages in low-molecular mass carbohydrates. Final products of hydrolysis were mainly monosaccharides and disaccharides, e.g. glucose and laminaribiose from curdlano-oligosaccharides, and glucose and cellobiose from cello oligosaccharides. Hydrolysis of cellopentaose was analysed by TLC. The activity of LamR was 0.75 U/mg or about 0.1% of the activity against laminarin.

LamR also displays a weak but significant activity against cellobiosyl-glucose G1-4G1-3Gr, the final product of lichenan hydrolysis by lichenases [1], suggesting again that LamR is able to cut, although only in low-molecular-mass carbohydrates, β-linkages which are not connected at the non-reducing end with 1,3-β-linkages (Fig. 6A–C).

LamR preparations purified from R. marinus and from recombinant E. coli cells showed identical properties. The enzymes from both sources displayed identical pH and temperature optima at pH 5.5 and 88°C. The enzyme is stable at 80°C. Incubation for two days at this temperature did not decrease the enzymatic activity. Incubation at 90°C decreased the enzymatic activity with a half-life (t1/2) of 27 min.

Characterisation of active-site mutants. Three acidic residues of bacterial lichenases (two Glu and one Asp residue) are involved in the enzymatic hydrolysis of β-1,4-linkages in mixed-linkage β-glucans [7, 8]. These residues are also invariant in several bacterial laminarinases (Fig. 1). Residues Glu129, Asp131 and Glu134 of LamR are essential for catalytic activity, as shown by the construction of active-site mutants. Substitutions leading to variant enzymes E129D, E129Q, D131E, D131N, E134D and E134Q affected significantly the enzymatic activity on laminarin and 1,3,1,4-β-glucan. Replacement of E129, D131 and E134 by their isosteric amide counterparts Q (in 129 and 134) and N (in 131) abolished any enzymatic activity. Substitution of the putative nucleophile E129 by another acidic residue had also a strong diminishing effect. The activity of variant enzyme E129D against 1,3,1,4-β-glucan or laminarin was more than three orders of magnitude lower than the wild-type activity. Reduction of catalytic activity was less pronounced, when the putative acid/base catalyst E134 and the assisting residue function D131 were replaced by D and E, respectively. In variant enzymes D131E and E134D 1,3,1,4-β-glucan-hydrolysing activity was significantly more reduced than the laminarinase activity.
Common features of bacterial laminarinases are a methionine residue inserted between the assisting D and the putative acid/base catalyst E and three additional amino acids within the active-site region of bacterial lichenases (Fig. 1). However, attempts to change the laminarinase to the lichenase phenotype by deletion of Met133 or PDN125−127 in LamR failed. Deletion of Met133 drastically reduced enzyme activity. Construction of double mutants with deleted PDN125−127 and Met133 residues abolished any hydrolysing activity.

If Met133 was replaced by Cys or Trp, hydrolysing activity against lichenan and laminarin was only slightly affected. The Met133A mutant display increased hydrolysing activity against mixed-linked oligosaccharide G1-4G1-3Gr as judged by TLC (Fig. 6C). A summary of all substitutions performed within the active-site region of LamR is presented in Table 1.

### Action pattern and subsite structure
Curdlan oligosaccharides ranging from DP4 to DP7 were labelled at the reducing end with the fluorescent dye 2-aminobenzamide and digested with LamR. Labelled cleavage products are visible after TLC. Curdlan oligosaccharides composed of 4−6 residues yielded only fluorescent laminaribiose (G1-3Gr). However, fluorogenic laminaribiose, laminarinribose and laminaritetraose were the hydrolysis products when labelled heptaose was incubated with LamR. This result suggests the existence of three or four subsites to the left (−1 to −III or −IV) and two subsites to the right (+I to +II) of the scissile bond of heptaose in the active site of LamR (Fig. 7).

Unlabelled curdlan oligosaccharides curdlano-oligosaccharides of different chain lengths ranging from DP2 to DP7 were also incubated with LamR. Enzyme activity against individual oligosaccharides increased steadily from DP2 to DP6. $k_{\text{cat}}$ was $33 \text{ s}^{-1}$ for DP5 while it increased to $137 \text{ s}^{-1}$ for DP6. No significant difference was found between DP6 and DP7 (143 s$^{-1}$) confirming again that six subsites might exist for substrate binding. $K_{\text{m}}$ values for DP5, DP6 and DP7 were similar (0.2−0.3 mM) indicating that affinity of the enzyme was not significantly affected by differences in chain length from 5 to 7.

### DISCUSSION
Representatives of bacterial 1,3-$\beta$ glucanases (laminarinases) including the enzyme of *R. marinus* and bacterial 1,3-1,4-$\beta$ glucanases (lichenases) are polysaccharide endo hydrolases with closely related and partially overlapping substrate specificities. According to weak but distinguishable similarities of their primary structures both types of glycosyl hydrolases have been classified as members of family 16 whilst other laminarinases without sequence similarity to bacterial lichenases have been classified into family 17 of glycosyl hydrolases [2, 3]. Sequence comparison reveals that the active-site residues E0103, D105 and E107 (numbering refers to *B. macerans* $\beta$-glucanase), involved in enzymatic catalysis of 1,3,1,4-$\beta$-glucanases (lichenases) of *B. macerans* and *B. licheniformis* [7], are invariant in other members of glycosyl hydrolyse family 16 including the laminarinase from *R. marinus* (Fig. 1). The *R. marinus* laminarinase (LamR) displays an exceptional high thermostability which is comparable with that of laminarinases isolated from other thermophilic and hyperthermophilic microorganisms [14, 15]. The enzyme cleaves soluble (laminarin) and insoluble (curdlan) 1,3-$\beta$-glucans and mixed-linkage 1,3-1,4-$\beta$-glucans generating DP3 and DP4 oligosaccharides as main products of hydrolysis. Homosaccharides and heterooligosaccharides containing 1,3-$\beta$-linkages and 1,4-$\beta$-linkages are also attacked by LamR. A total of five or six substrate-binding sites could be proposed for LamR, on the basis of kinetic studies performed with oligosaccharides of increasing degree of polymerisation. Laminarhexaose (G1−3G3), is readily hydrolysed by LamR and no increase in the rate of hydrolysis was found when the equivalent heptaose was used as substrate. This result suggests a number of subsites less than seven. The fluorogenic heptaose was hydrolysed to G1-3G1-3G1-3G1-3G1-3G1, indicating that no preference in the cleavage-site position exists. Results obtained with unlabelled oligosaccharides with DP from 4 to 7 confirm that the enzyme binds at several positions but preferentially to the inner core of the curdlano-oligosaccharides as described for the *Aspergillus fumigatus* 1,3-$\beta$ glucanase [30]. Hydrolysis of

**Table 1. Characteristics of wild type and mutant LamR enzymes.** n.m., not measurable. All measurements have been performed in triplicate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$</th>
<th>$K_{\text{m}}$</th>
<th>Activity</th>
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<tbody>
<tr>
<td></td>
<td>$\beta$-glucan</td>
<td>laminarin</td>
<td>$\beta$-glucan/laminarin</td>
</tr>
<tr>
<td>Wild type</td>
<td>2199 ± 1139</td>
<td>656 ± 12</td>
<td>1.3 ± 0.1</td>
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<td>Active site residues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E129Q</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>E129D</td>
<td>1.7 ± 0.5</td>
<td>0.5 ± 0.2</td>
<td>3.3 ± 1.5</td>
</tr>
<tr>
<td>D131N</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>D131E</td>
<td>7.1 ± 3.0</td>
<td>20 ± 4.0</td>
<td>6.5 ± 1.1</td>
</tr>
<tr>
<td>E134D</td>
<td>10.4 ± 1.5</td>
<td>45.1 ± 5.0</td>
<td>n.m.</td>
</tr>
<tr>
<td>E134Q</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>Deletions of residues, only present in laminarinases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔPDN 125−127</td>
<td>0.006 ± 0.006</td>
<td>0.002 ± 0.002</td>
<td>n.m.</td>
</tr>
<tr>
<td>ΔM133</td>
<td>1.1 ± 3.3</td>
<td>3.1 ± 1.0</td>
<td>n.m.</td>
</tr>
<tr>
<td>ΔPDN + ΔM133</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>Substitutions in M133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M133A</td>
<td>1695 ± 101</td>
<td>328 ± 10</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>M133C</td>
<td>1398 ± 57</td>
<td>416 ± 3</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>M133W</td>
<td>1058 ± 15</td>
<td>532 ± 14</td>
<td>5.2 ± 0.1</td>
</tr>
</tbody>
</table>
cose proved that in mixed-linkage carbohydrates and next to the group of family 1 glycosyl hydrolases, LamR also cleaves 1,4-β-linkages which are not connected with 1,3-β-linkages at the non-reducing end. This was demonstrated by the hydrolysis of cellulosic oligosaccharides and by the cleavage pattern of the trisaccharide G1-4G1-3G, yielding glucose and laminariobiose but not cellobiose.

There are two lines of evidence supporting the catalytic role of LamR residues E129, D131 and E134 and their relatedness to E103, D105 and E107 in \textit{B. macerans} 1,3,1,4-β-glucanase. Firstly, the results of activity measurements of mutants substituted in the three acidic residues equivalent to the active-site residues in \textit{Bacillus} β-glucan hydrolyases [7, 8, 28] demonstrated their importance for laminarinase activity. Isosteric replacements E129Q, D131N and E134Q resulted in mutant proteins without detectable activity, suggesting the necessity of negatively charged side chains (carboxylic groups) on the putative nucleophile E129, the general catalytic acid E134 and the assisting residue D131 probably stabilising the nucleophile. Substitution of E129 by D reduced the remaining enzyme activity more strongly than the same substitution in the putative general acid E134.

Secondly, the active site of \textit{Trichoderma} enzyme CBHI shows structural similarity to the active-site region of \textit{Bacillus} 1,3,1,4-β-glucanase. Superposition of active-site residues of CBHI and lichenase suggests conservation of the three acidic residues and also of two W residues involved in substrate binding of both enzymes [31]. In \textit{B. macerans} β-glucanase active-site residues are located within a β-strand formed by amino acid residues D102–L109. The acidic residues E103, D105 and E107 point toward the cleft, whereas D102, N104 and N106 are oriented away from the active-site cleft, excluding the possibility of their direct participation in enzymatic hydrolysis [28]. E103 and E107 were assigned to be directly involved in cleavage of the 1,4-β-glycosidic bond by functioning as the catalytic nucleophile and as the general acid, respectively. According to the three-dimensional model, W101 and Asp105 are assisting enzymatic reaction by forming hydrogen bonds via their side chains to Glu103 in order to position properly the nucleophile [7]. The carboxylic side chains of CBHI E212, D214 and E217 which are equivalent to E103, D105 and E107 in \textit{B. macerans} 1,3,1,4-β-glucanase are located within a β-strand formed by amino acid residues E212–A218 and were shown to catalyse the double-displacement reaction in \textit{Trichoderma} CBHI enzyme. As observed in \textit{Bacillus} 1,3,1,4-β-glucanase and other retaining enzymes, the distance between the two carboxylate groups of the suggested general acid or proton donor (E217) and the nucleophile (E212) in CBHI is in the range of 5–6 Å [32]. Changing these residues to their isosteric amide counterparts results in significant but different reduction of CBHI activity [31]. Despite of differences in the primary structure of the active-site regions of \textit{Bacillus} lichenase and \textit{Trichoderma} CBHI which is especially reflected by the presence of an additional amino acid residue W216, the central folding motif (β-sandwich) including the catalytic triad E212, D214 and E217 is structurally well conserved in both enzymes. The three-dimensional model established for the active-site region of CBHI demonstrates that insertion of W216 does not affect orientation of the putative proton donor E217 whose active carboxy side chain points to the active-active-site cleft [32]. The primary structure of LamR also contains an additional amino acid residue, Met133, which is equivalent to CBHI Trp216, suggesting that the architecture of the active-site region could be structurally related to CBHI where two adjacent residues, I215 and W216, are pointing away from the cleft (Fig. 8).

![Fig. 7. TLC analysis of dye-labelled curdlan oligosaccharides digested by LamR. (G1-3G)\_\_G-2-AB were incubated for 15 min at 65°C with enzyme, 550 U/ml. The digest was separated by TLC and visualised under ultraviolet at 305 nm. k\textsubscript{m} values determined for unlabelled curdlan oligosaccharides (DP5 to DP7) are shown for comparison. The putative cleavage sites and possible orientations to the subsite structure deduced from the detected hydrolysis products are presented in the right part of the figure.](image-url)
Fig. 8. Structural relatedness within the active-site regions of β-glucanases consisting of the catalytic triads E103 D105 E107 (B. macerans 1,3 1,4 β-glucanase) and E212 D214 E217 (Tr. reesei CBHI). The schematic drawing of the active site sequence of LamR depicted in the upper part of the figure was deduced from protein modelling data. Replacements of M213 and W216 by I213 and M216 in CBHI reveal identical sequences of CBHI and LamR within the two catalytic E residues. Modelling of the CBHI (1cel.pdb, P00725) variant with substitutions M213I and W216M was performed by automated protein modelling (method ProMod2, [36]) using the Swiss Model Server. The molecular graphic program RasMol v2.6 (R. Sayle, Biomolecular Structure, Glaxo Research and Development, Greenford, Middlesex, UK) was used to visualise the structure.

Deletion of Met133 leads to laminarinase with drastically reduced activity demonstrating its importance for structural integrity. Mutants M133A, M133C, M133W were found to tolerate such substitutions and displayed near wild-type activity when incubated with laminarin or β-glucan.

The results presented here support the idea that the acquisition of distinct substrate specificities in the evolution of related β-glucan hydrolases does not require the recruitment of novel catalytic amino acids but rather differences in their positioning within the active site and/or changes in substrate-binding residues as was also shown in barley laminarinases and lichenases [33, 34]. However, the elucidation of the three-dimensional structure of LamR and a detailed comparison with that of Bacillus 1,3-1,4-β-glucanase [7, 28] is necessary to verify the conclusions derived from the site-directed mutagenesis and sequence comparison which are presented here.

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REFERENCES


