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Enzymatic synthesis of 4-methylumbelliferyl (1→3)-β-D-glucooligosaccharides—new substrates for β-1,3-1,4-D-glucanase

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Abstract

The transglycosylation reactions catalyzed by β-1,3-D-glucanases (laminaranases) were used to synthesize a number of 4-methylumbelliferyl (MeUmb) (1→3)-β-D-gluco-oligosaccharides having the common structure [β-D-Glc*p*-(1→3)]$_n$-β-D-Glc*p*-MeUmb, where $n = 1–5$. The β-1,3-D-glucanases used were purified from the culture liquid of *Oerskovia* sp. and from a homogenate of the marine mollusc *Spisula sachalinensis*. Laminaran and curdlan were used as (1→3)-β-D-glucan donor substrates, while MeUmb-β-D-glucoside (MeUmbGlc*p*) was employed as a transglycosylation acceptor. Modification of [β-D-Glc*p*-(1→3)]$_2$-β-D-Glc*p*-MeUmb (MeUmbG$_3$) gives 4,6-*O*-benzylidene-D-glucopyranosyl or 4,6-*O*-ethylidene-D-glucopyranosyl groups at the non-reducing end of artificial oligosaccharides. The structures of all oligosaccharides obtained were solved by ^{1}H and ^{13}C NMR spectroscopy and electrospray tandem mass spectrometry. The synthetic oligosaccharides were shown to be substrates for a β-1,3-1,4-D-glucanase from *Rhodothermus marinus*, which releases MeUmb from β-di- and β-triglucosides and from acetal-protected β-triglucosides. When acting upon substrates with d.p. > 3, the enzyme exhibits an endolytic activity, primarily cleaving off MeUmbGlc*p* and MeUmbG$_2$.

Keywords: 4-Methylumbelliferyl (1→3)-β-D-glucoside; β-1,3-1,4-D-Glucanase; *Rhodothermus marinus*; Transglycosylation; Laminaranase

1. Introduction

Fluorogenic and chromogenic saccharides are convenient and widely used substrates to study the kinetic properties of glycosyl hydrolases and transglycosylases, since many exo- and endo-glycosidases are able to release chromophoric or fluorophoric aglycon groups from these substrates. This allows the development of kinetic assays which are both more sensitive and more simple to analyse when compared with assays where mono- or oligosaccharides are detected as products of hydrolysis.[1,2] Synthetic fluorogenic mono- and oligosaccharides have also been used as modified ligands to study lectins.[3] Commercially available 4-methylumbelliferyl-, *o*-nitrophenyl- and *p*-nitrophenyl (PNP) glycosides used as substrates for exo-glycosidases are, as a general rule, products of relatively high-yielding organic synthesis.[4,5] Obtaining fluorogenic and chomogenic oligosaccharides, substrates for endo-glycosidases,[6] is essentially a more difficult task for organic chemistry. Classical synthetic approaches suffer the drawback, however, that they require numerous protection and

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deprotection steps in addition to those in which glycosidic bonds are formed.[7,8] Considerable effort has gone into studying the application of the transglycosylating ability of retaining glycosidases as an alternative approach for the synthesis of chromo- and fluorogenic oligosaccharide substrates.[9–11] One potential limitation with this approach, however, is that the products of transglycosylation may themselves be hydrolysed by the enzyme during the course of the reaction. The introduction of glycosynthases, mutant glycosidases which lack a catalytic nucleophile and are thus incapable of carrying out substrate hydrolysis but which catalyse transglycosylation in the presence of glycosyl fluoride donors, has overcome this problem for the synthesis of a number of β-[12,13] and α-aryl[14] oligosaccharides.

In this work, we used the inherent transglycosylating activity of two endo-β-1,3-D-glucanases (laminaranases), one from the bacterium *Oerskovia* sp. and one from the marine mollusc *S. sachalinensis*, to synthesize β-D-gluco-oligosaccharides with the common structure of $[\beta\text{-D-Glc-}(1\rightarrow3)]_n\text{-}\beta\text{-D-Glc-MeUmb}$, where $n = 1–5$, as novel substrates for analysing the β-1,3-1,4-D-glucanase (β-1,3-1,4-D-glucan glucohydrolase; E.C. 3.2.1.73, β-1,3-1,4-glucanase) from *Rhodothermus marinus*. This thermophilic β-1,3-1,4-D-glucanase belongs to glycosyl hydrolase family 16, which is comprised mainly of endo-acting β-glucanases. The range of substrates acted upon by enzymes in this family includes (1→3)-β-D-glucans, such as laminaran, or mixed linkage (1→3),(1→4)-β-D-glucans like lichenan and barley glucan.[15] Although the enzymatic properties, DNA sequence and transglycosylation activity of this enzyme have been described recently,[15,16] elucidating the structure–function aspects of the transglycosylation activity requires more detailed study, which is made possible only with non-natural substrates. The novel MeUmb substrates presented in this study should prove generally useful for future comparative studies of wild-type and mutant β-1,3-1,4-D-glucanases from a variety of sources.

2. Results and discussion

An extracellular β-1,3-D-glucanase (laminaranase) possessing transglycosylation activity was isolated from culture filtrates of *Oerskovia* sp. The partially purified enzyme contained less than 0.1% each of β-glucosidase, β-1,6-glucanase and cellulase activities, thus permitting use of the preparation for the enzymatic synthesis of (1→3)-β-D-glucosides. The enzyme preparation was found to be stable at 37 °C in the pH range of 4–7 (20 mM sodium acetate or 20 mM sodium phosphate buffers) for at least 72 h.

The enzyme exhibits transglycosylation activity when curdlan [(1→3)-β-D-glucan] was used as a donor and MeUmbGlc*p* as an acceptor. The transglycosylation ability of the enzyme was studied by analysing MeUmb-containing oligosaccharides with thin-layer chromatography (TLC) and analytical reverse-phase high performance liquid chromatography (HPLC). Separated oligosaccharides containing the fluorophoric group were isolated from the reaction mixture by gel-permeation chromatography followed by a second purification step using reverse-phase HPLC. Individual fractions obtained by HPLC were more than 95% pure and were further characterized with electrospray ionisation mass spectrometry, ^{1}H and ^{13}C NMR.

ESI–TOF MS data recorded for the $(M+Na)^+$ ion, which was formed to the exclusion of any other molecular adducts under the experimental conditions, indicate that the observed masses correlate well with the calculated monoisotopic masses of the expected products (Table 1). In order to provide further evidence for the proposed structures, tandem mass spectra (MS/MS) were recorded for each compound in Table 1. The benzylidene-protected oligosaccharides gave excellent fragmentation patterns, which allowed the confirmation of the stoichiometry of glucosyl, methylumbelliferyl and benzylidene residues (Fig. 1). However, none of the unprotected MeUmb β-laminara-oligosaccharides, with the exception of the laminaratriose substrate, produced a complete Y- or B-type fragment ion series under a variety of CID conditions studied. Instead, the only predominant fragment ions observed in MS/MS spectra of these compounds were those resulting from the neutral loss of methylumbelliferone (7-hydroxy-4-methylcoumarin) to yield a B-type ion and of a MeUmb-dehydroglucosyl residue to yield a C-type ion (Fig. 2). Generation of the observed C_1 ions may occur sequentially by β elimination from the B_n ion ($n =$

Table 1
Calculated and observed monoisotopic molecular masses of sodium adducts using externally calibrated ESI–TOF MS

Product	Calculated	Observed	Error (ppm)
$MeUmbG_2$	$C_{22}H_{28}NaO_{13}$: 523.1428	523.1535	20
$MeUmbG_3$	$C_{28}H_{38}NaO_{18}$: 685.1956	685.2074	17
$MeUmbG_4$	$C_{34}H_{48}NaO_{23}$: 847.2484	847.2820	40
$MeUmbG_5$	$C_{40}H_{58}NaO_{28}$: 1009.3012	1009.3408	39
$MeUmbG_6$	$C_{46}H_{68}NaO_{33}$: 1171.3541	1171.4065	45
$BnzMeUmbG_3$	$C_{35}H_{42}NaO_{18}$: 773.2269	773.2299	3.8
$BnzMeUmbG_4$	$C_{41}H_{52}NaO_{23}$: 935.2797	935.2888	9.7
$BnzMeUmbG_5$	$C_{47}H_{62}NaO_{28}$: 1097.3325	1097.3329	0.36

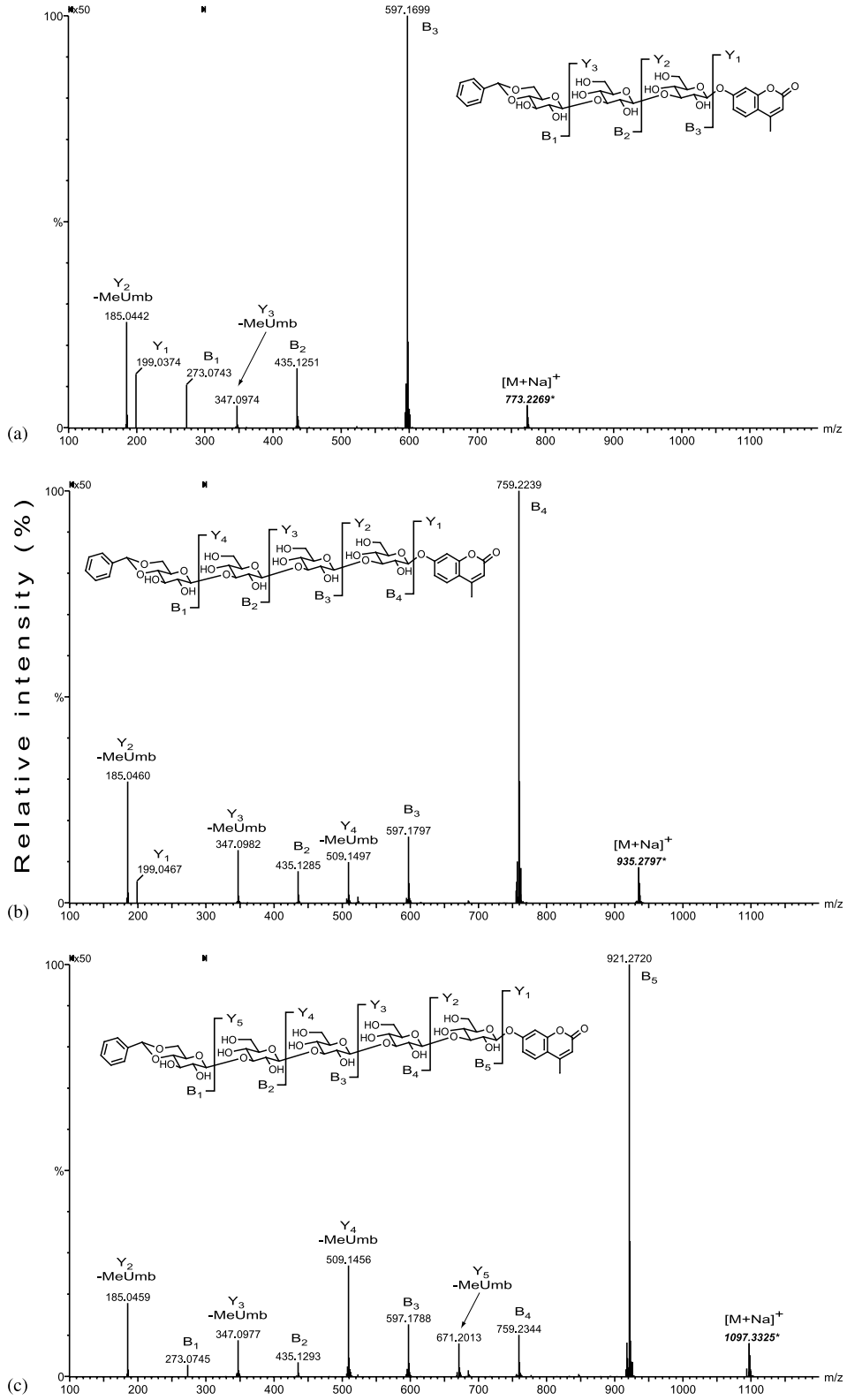

Fig. 1. CID MS/MS spectra of benzylidene-protected MeUmb-(1→3)-glucooligosaccharides. (A) Bzl-Glc_3-MeUmb; (B) Bzl-Glc_4-MeUmb; (C) Bzl-Glc_5-MeUmb. Labels in bold italics marked with an asterix indicate peaks used for 'lock mass' correction.

number of glucose units in the parent ion) and provides additional evidence for a (1→3)-β linkage between the first and second glucosyl residues. By comparison, β(1→4)-linked MeUmb gluco-oligosaccharides give complete B-ion series when subjected to CID under similar conditions [Brumer, Neustroev, and coworkers,

unpublished]. To further characterize these fragment ions, 'virtual' MS^3 experiments were performed on the MeUmb-Glc_5 compound by subjecting the $(M+Na)^+$ adduct to higher cone voltages sufficient to cause in-source fragmentation. Fragment ions with identical masses to those observed previously in the CID spectra were produced, and these were individually selected with the quadrupole to generate CID MS/MS spectra. As shown in Fig. 3, the fragment ions observed in spectrum B at *m/z* 833.3 and *m/z* 689.2 yield complete Y- and B-type ion series in addition to ions resulting from cross-ring fragmentation. The observation of these latter ions is most certainly a consequence of the considerably higher energy imparted on the parent ion in the source region of the mass spectrometer, since the collision energy is similar (approx 50 V) to that used to obtain

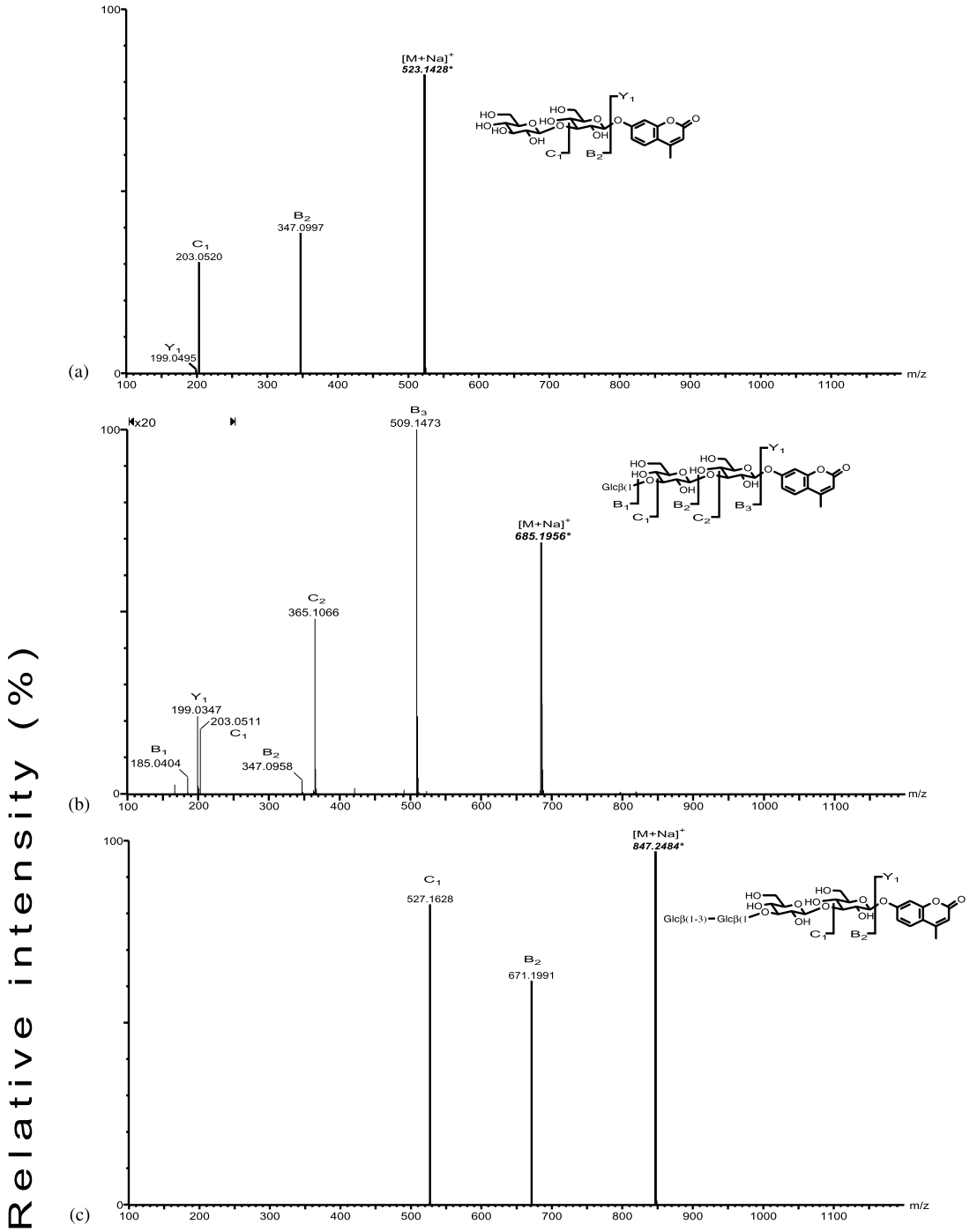

Fig. 2. CID MS/MS spectra of MeUmb-(1→3)-glucooligosaccharides. (A) Glc_2-MeUmb; (B) Glc_3-MeUmb; (C) Glc_4-MeUmb; (D) Glc_5-MeUmb; (E) Glc_6-MeUmb. Labels in bold italics marked with an asterix indicate peaks used for 'lock mass' correction.

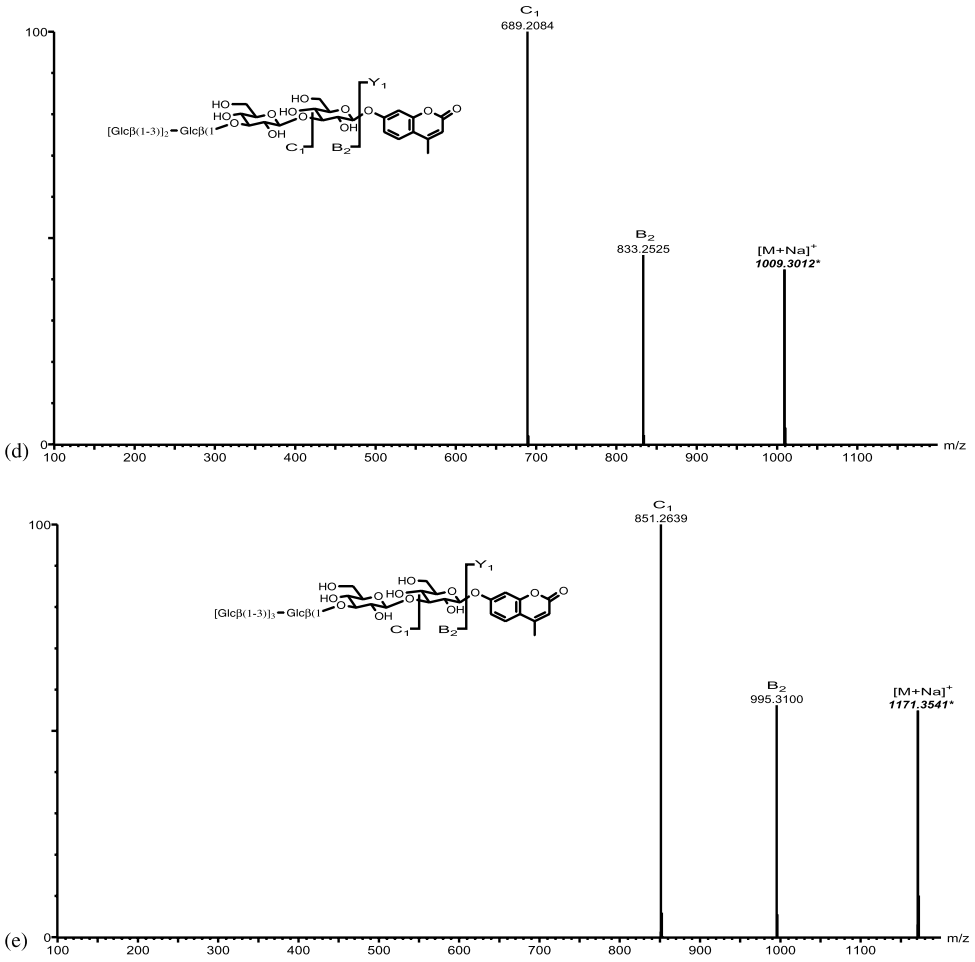

Fig. 2 (*Continued*)

CID of the $(M+Na)^+$ ion under lower cone voltage conditions. Taken together, the monoisotopic mass value obtained in spectrum A and the tandem MS data in spectra B–D (Fig. 3) provide conclusive evidence of the stoichiometry of glucosyl and methylumbelliferyl residues in the molecule. Similar exhaustive fragmention analysis was not carried out for other molecules in the glycan series, but the correctness of the ion assignments in Fig. 2 may be inferred by analogy.

The exact numbers of glucosyl residues were also derived from ^1H and ^{13}C NMR spectra, which contain from two to four characteristic, low-field signals for anomeric protons and carbon atoms (Table 2). The ^{13}C NMR spectra contain also the same numbers of high-field signals for C-6, which can be easily identified by the DEPT technique. Analysis of vicinal coupling constants of ^1H spectra shows that all glucose units are hexopyranoses with equatorial hydroxy groups, namely β-glucopyranose. Anomeric configurations of all the monosaccharide residues were confirmed by the low-field chemical shift values for the anomeric carbon atoms (Table 3). In addition, the (1→3)-type of the glycosidic linkages in the oligosaccharides obtained was determined by analysing the positions of the low-field signals for C-3 in the ^{13}C NMR spectra. Data from 2D NMR spectroscopy (COSY, ^1H–^{13}C-heteronuclear correlations, and NOESY) establish the structures of the oligosacharides unequivocally. After enzymatic synthesis with *Oerskovia* β-1,3-D-glucanase, a set of (1→3)-D-Glc_nMeUmb oligosaccharides with $n=2$–4 was obtained from curdlan. The yield for each of these oligosaccharides is shown in Table 4. Thus, only (1→3)-β-linked MeUmb β-laminara-oligosaccharides with d.p. 2–4 were produced as a result of the regio- and stereoselective synthesis performed with the aid of the transglycosylation ability of the endo-β-glucanase from *Oerskovia* sp. When laminaran from *Laminaria digitata* was also used as a glycosyl donor of β-laminara-oligosaccharides for the transglycosylation reaction catalyzed by the β-1,3-D-glucanase from *Oerskovia* sp., TLC and HPLC analyses of these products showed similar sets of products ranging from d.p. 2 to 4. However, the use of laminaran as a source of β-oligosaccharides for the enzymatic synthesis is less preferable due to presence of about 10% β-(1→6)-linked glucan branches on the main chain. As a result, the

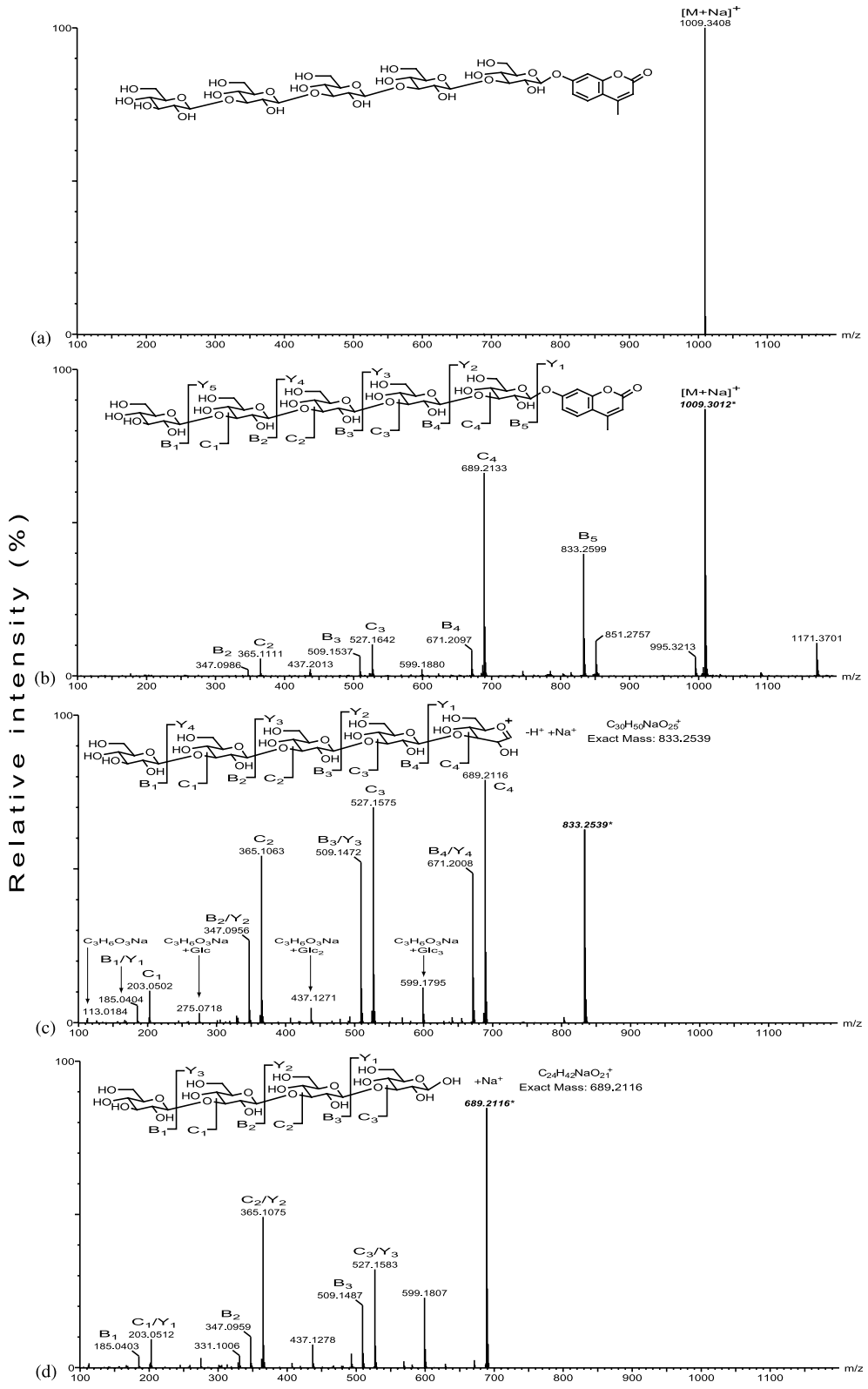

Fig. 3. CID MS/MS spectra of in-source fragment ions of the Glc_5-MeUmb sodium adduct. (A) TOF MS spectrum of Glc_5-MeUmb with cone voltage set at 80 V; (B) TOF MS spectrum of Glc_5-MeUmb with cone voltage set at 160 V; (C) MS/MS spectrum of in-source fragment 833.25 (cone 160 V, collision energy 60 V); (D) MS/MS spectrum of in-source fragment 689.21 (cone 160 V, collision energy 60 V). Labels in bold italics marked with an asterix indicate peaks used for 'lock mass' correction.

Table 2
Chemical shifts obtained from ^{1}H NMR spectra of MeUmb β-laminaraoligosaccharides

Product		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
$MeUmbG_2$								
β-D-Glc*p*-(1→3)	B	5.130	3.805	3.881	3.636	3.695	3.977	3.817
β-D-Glc*p*	A	4.803	3.398	3.551	3.429	3.517	3.946	3.742
$MeUmbG_3$								
β-D-Glc*p*-(1→3)	C	5.196	3.826	3.908	3.647	3.722	3.986	3.819
β-D-Glc*p*-(1→3)	B	4.851	3.595	3.801	3.538	3.537	3.953	3.764
β-D-Glc*p*	A	4.770	3.374	3.538	3.416	3.494	3.927	3.727
$MeUmbG_4$								
β-D-Glc*p*-(1→3)	D	5.220	3.829	3.909	3.647	3.728	3.982	3.815
β-D-Glc*p*-(1→3)	C	4.848	3.593	3.808	3.530	3.525	3.950	3.761
β-D-Glc*p*-(1→3)	B	4.811	3.565	3.784	3.530	3.531	3.929	3.756
β-D-Glc*p*	A	4.759	3.363	3.530	3.409	3.486	3.921	3.721

Table 3
Chemical shifts obtained from ^{13}C NMR spectra of MeUmb β-laminaraoligosaccharides

Product		C-1	C-2	C-3	C-4	C-5	C-6
$MeUmbG_2$							
β-D-Glc*p*-(1→3)	B	101.95	75.03	86.51	70.34	78.31	62.90
β-D-Glc*p*	A	105.32	75.96	78.04	72.07	78.51	63.20
$MeUmbG_3$							
β-D-Glc*p*-(1→3)	C	101.96	75.09	86.35	70.31	78.32	62.92
β-D-Glc*p*-(1→3)	B	105.04	71.76	86.77	70.63	78.13	63.19
β-D-Glc*p*	A	105.32	75.94	76.05	72.06	78.49	63.19
$MeUmbG_4$							
β-D-Glc*p*-(1→3)	D	101.95	75.09	86.28	70.29	78.31	62.88
β-D-Glc*p*-(1→3)	C	105.03	75.80	86.52	70.58	78.12	63.15
β-D-Glc*p*-(1→3)	B	105.00	75.72	86.71	70.58	78.09	63.15
β-D-Glc*p*	A	105.28	75.92	78.02	72.04	78.47	63.15

products of the reaction had the same length but differed in configuration of β-glucosidic bonds. These products were only poorly separated by HPLC with a relatively low yield of approx 40%, which limited the amount of MeUmb (1→3)-β-glucosides which could be obtained (Table 4). The pH optimum of the transglycosylation reaction lies between 5 and 6.5 (Fig. 4), however, the ratio of the individual (1→3)-β-glucooligosaccharides formed in the course of the reaction is pH independent.

With the *Oerskovia* sp. enzyme, the lowest yield obtained was for $MeUmbG_4$ (Table 4) and MeUmb-containing β-D-glucosides of higher polymerization were detected in trace quantities. Since higher oligosaccharides with a d.p. of 4 or greater would serve as useful substrates for kinetic studies of the β-1,3-1,4-D-glucanases,[17] enzymatic synthesis was explored with the endo-1,3-β-D-glucanase (laminaranase) from the marine mollusc *S. sachalinensis*. The selection of this enzyme was based upon its recent successful application in the synthesis of PNP β-laminara-oligosaccharides.[18] Trans-

Table 4
Yields of products from enzymatic synthesis catalyzed by β-1,3-D-glucanases from *Oerskovia* sp. and mollusc *S. sachalinensis* using laminaran and curdlan as donors

Product	*Oerskovia* β-1,3-D-glucanase		Mollusc β-1,3-D-glucanase	
	Donor, yield (%)		Donor, yield (%)	
	Curdlan	Laminaran	Curdlan	Laminaran
$MeUmbG_2$	30	21	12	30
$MeUmbG_3$	25	17	8	20
$MeUmbG_4$	8	4	Traces	15
$MeUmbG_5$	traces	traces	traces	8
$MeUmbG_6$	traces	traces	traces	6

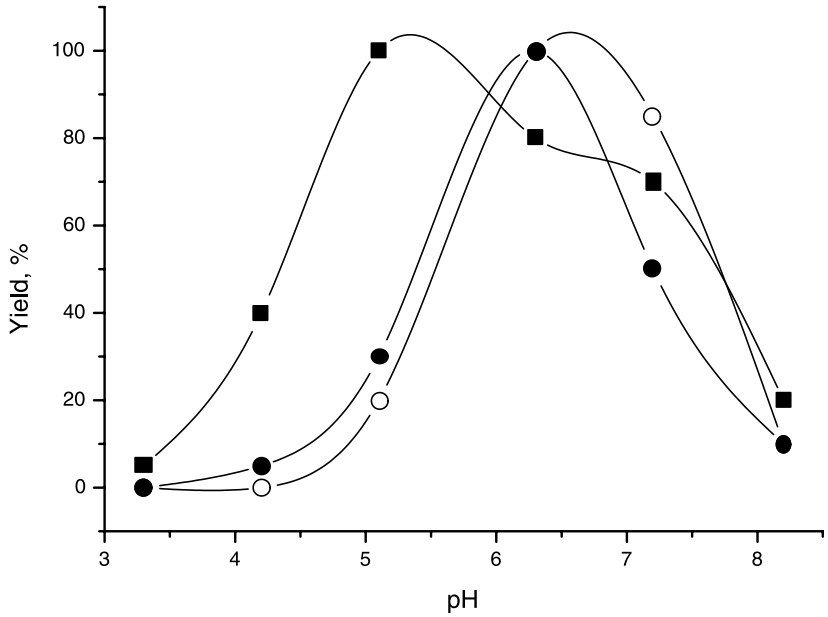

Fig. 4. The pH-optima of the *Oerskovia* endo-β-glucanase-catalyzed reactions: ○, product formation during the hydrolysis of laminaran; ● and ■, product formation during the transglycosylation reactions with laminaran and curdlan as donors, respectively. The analyses were done on a reversed-phase Waters Hypersil ODS column using a linear gradient (0–90%) of MeCN in water. The amounts of products formed were calculated by integrating the corresponding chromatographic peaks.

Table 5
Kinetic parameters of hydrolysis of MeUmb-containing β-D-glucooligosaccharides by β-1,3-1,4-D-glucanase from *R. marinus*

Substrate	K_m (μmol)	$10^5 \times V_{max}$ (μmol/(min μg))
$MeUmbG_2$	103.1	20.2
$MeUmbG_3$	28.6	25.4
$BnzMeUmbG_3$	32.5	15.8
$EtMeUmbG_3$	94.3	5.4

glycosylation with the β-1,3-D-glucanase from *S. sachalinensis* lead to the formation of products with a d.p. of 5 and 6 in quantities suitable for enzymatic studies (Table 5). Minor quantities of MeUmb-β-laminara-oligosaccharides with d.p. more than 6 were detected in the reaction mixtures by TLC and HPLC, however, these were not pursued due to their low yields ($<2\%$ by analytical HPLC analysis).

It should be noted that microbial glycosyl hydrolases are traditionally considered to be good tools for the enzymatic synthesis of glycosides due to their high pH- and thermal-stability, and because they are readily produced in large scale.[19] In comparison, eukaryotic glycosyl hydrolases are used less frequently.[20] In this study, the combined application of the microbial *Oerskovia* and mollusc endo-β-1,3-D-glucanases proved effective in the production of a set of MeUmb-β-D-glucooligosaccharides due to their complimentary substrate specificities and product distributions. The enzymatic synthesis reported herein has certain advantages in comparison to the traditional synthetic route which has been presented for oligosaccharides possessing the common structure [β-D-Glc*p*-(1→4)]$_n$-β-D-Glc*p*-(1→3)-β-D-Glc*p*-MeUmb ($n=1$–3).[8] The enzymatic approach does not require as many individual reaction steps and, as a result, has a higher total yield of the final products. Most notably, the enzymatic synthesis permits one to obtain glucooligosaccharides with higher d.p., such as $MeUmbG_5$ and $MeUmbG_6$. Additionally, the pure organic synthetic route to produce MeUmb-β-laminara-oligosaccharides requires sizable quantities of individually purified oligosaccharides derived from laminaran as starting materials. Such oligosaccharides can only be produced from laminaran or curdlan by enzymatic digestion or acid hydrolysis followed by chromatographic purification, which is both low yielding and tedious.[21,22]

All the synthesized MeUmb oligosaccharides were tested as substrates for the β-1,3-1,4-D-glucanase from *R. marinus*. As determined by HPLC and TLC analyses, the enzyme releases MeUmb from $MeUmbG_2$ and $MeUmbG_3$ (kinetic parameters for these reactions are shown in Table 5), while the reaction of the enzyme with MeUmb oligosaccharides of higher d.p. (4–6) gives a number of products. Table 6 shows the ratio of MeUmb-containing products released during enzymatic hydrolysis. The data indicate that $MeUmbG_2$ and $MeUmbG_3$ are ideal substrates for precise kinetic studies of the enzyme due to a higher sensitivity of detection when compared with the methods of detecting

Table 6
Yields [a] of products from hydrolysis of MeUmb β-laminaraoligosaccharides by β-1,3-1,4-D-glucanase from *R. marinus*

Substrate	Products (mol/mol of MeUmb products) [b]					
	MeUmb	MeUmbG	$MeUmbG_2$	$MeUmbG_3$	$MeUmbG_4$	$MeUmbG_5$
$MeUmbG_2$	0.24		0.76			
$MeUmbG_3$	0.34			0.65		
$MeUmbG_4$	0.22	0.14	0.03	0.11	0.5	
$MeUmbG_5$	0.23	0.09	0.005	0.009	0.06	0.6
$MeUmbG_6$	0.345	0.083	0.005	0.007	0.012	0.44

[a] The yields given are an average of at least 3 determinations.
[b] Experimental error is ±5% of values given.

reducing sugars released during hydrolysis[23,24] or the use of alternative substrates such as Congo Red dye-labeled β-D-glucans.[25] The ability to liberate MeUmb from $MeUmbG_2$ and $MeUmbG_3$ is most likely a common feature in mode of action of β-1,3-1,4-D-glucanases and β-1,3-D-glucanases. For example, MeUmb is the only chromophoric product in hydrolysis of these substrates by a β-1,3-D-glucanase from *Trichoderma* sp. (data not shown). A similar mode of action was also reported for a β-1,3-1,4-D-glucanase from *Bacillus licheniformis*.[26] Consequently, we may suppose that the synthesized substrates can be used broadly as effective tools in the investigation of glycosidase activities in different members of glycosyl hydrolase family 16 which act upon (1→3)-β-glucans and mixed linked (1→3),(1→4)-β-D-glucans.

Protected MeUmb oligosaccharides with 4,6-*O*-benzylidene-D-glucopyranosyl or 4,6-*O*-ethylidene-D-glucopyranosyl groups ($BzlG_3$MeUmb and EtG_3MeUmb) were also shown to serve as substrates for the *R. marinus* β-1,3-1,4-D-glucanase. As with the unprotected analogues, the enzyme releases MeUmb yielding $BzlG_3$ and EtG_3 as hydrolysis products. Interestingly, the K_M and V_{max} values for these substrates (Table 5) are essentially the same as those obtained for unprotected $MeUmbG_2$ and $MeUmbG_3$ oligosaccharides and β-D-glucans, laminaran and curdlan, that was reported earlier.[15] The utility of these substrates for the screening of bacterial strains for the presence of endo-β-1,3-1,4-D-glucanase activity is directly indicated, since protection of the non-reducing end of the substrate discriminates against β-glucosidase activity. It has previously been demonstrated that fluorescent and chromogenic substrates significantly improve the sensitivity of microtiter plate-based screening assays of glycosyl hydrolases.[27,28] The favorable kinetic parameters observed for the hydrolysis of protected MeUmb-β-laminarabioside and MeUmb-β-laminaratrioside by *R. marinus* β-1,3-1,4-D-glucanase supposes a successful application of these substrates in such studies including detection of β-glucanase in the presence of β-glucosidase.

3. Experimental

3.1. Materials

Laminaran from *L. digitata*, barley (1→3),(1→4)-β-glucan, cellulose, *p*-nitrophenyl β-D-glucosides were from Sigma Chemical Co. (St. Louis, MO, USA). Curdlan from *Alcaligenes faecalis* was kindly donated by Professor Irwin J. Goldstein, University of Michigan, Ann Arbor, USA. All reagents were of analytical or research grade. 4-Methylumbelliferyl β-D-glucopyranoside was synthesized according to Ref. 5.

3.2. Analytical methods

Protein concentrations were measured in accordance with the Lowry procedure using BSA as a standard.[29] The protein concentration of β-1,3-1,4-D-glucanase from *R. marinus* was determined using an absorption coefficient at 282 nm of $A^{1\%}$ 32.38 mL/(mg cm).[16] Optical rotations were determined with a JASCO DIP-360 polarimeter. Qualitative TLC analysis of oligosaccharide substrates and products of enzymatic hydrolysis were performed using Kieselgel 60 plates from Merck (Darmstadt, Germany) with a mobile phase of 2:1:1 EtOAc–AcOH–water. All ^1H and ^{13}C NMR spectra were recorded with an AMX-500 Bruker spectrometer (^1H at 500.13 MHz, ^{13}C at 125.13 MHz) in D_2O at ambient temperature with acetone as an internal reference standard (δ_H 2.225; δ_C 31.45). One-dimensional ^1H, NOE and ^{13}C spectra and phase-sensitive two-dimensional spectra (COSY-DQF, NOESY and ^1H–^{13}C heteronuclear correlations) were recorded using standard pulse programs at 20 and 50 °C. Data were analysed using the XWINNMR software package (Bruker, Germany). The assignment of individual sugar residues was based on two-dimensional phase-sensitive COSY data. Monosaccharide sequence assignments were based on NOE cross-peaks between the anomeric proton and proton at the substitution position in NOESY experiments using a mixing time of 180 ms. Assignment of ^{13}C signals was based on the proton–carbon correlations

observed by $^{1}H-^{13}C$ correlation spectroscopy. Determination of the anomeric configuration of each monosaccharide residue was based on the observed chemical shifts of the anomeric (C-1) protons in one-dimensional ^{1}H spectra and the $J_{1,2}$ coupling constants.

Positive-ion mass spectra were recorded on a Micromass Q-TOF2 orthogonal acceleration quadrupole/time-of-flight mass spectrometer (Micromass, Manchester, UK). With the TOF analyser operating in single-reflectron 'V' mode, typical peak resolution on singly-charged sodiated carbohydrate adducts was 10,000–12,000 FWHM in TOF MS mode. 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 Pr^{i}OH–water. Solutions of MeUmb glucooligosaccharides were infused into the mass spectrometer through a nanoflow ion source at a concentration of 0.03 mg/mL in 1:1 MeOH–water containing 0.5 mM NaCl by a syringe pump at 500 nL/min. During the analysis of benzylidene-protected MeUmb glucooligosaccharides (0.002–0.005 mg/mL, in 1:1 MeOH–water containing 0.5 mM NaCl, flow rate 5 μL/min) an electrospray ion source (desolvation temp. 150 °C) was mounted on the instrument. The nanospray source capillary voltage optimized at 3.0–3.5 kV and the electrospray source voltage was maintained at 3.0 kV. The choice of source had no significant effect on the ionization of the analytes studied. The cone voltage was varied during TOF MS acquisition to optimise the intensity of the $(M+Na)^{+}$ ion signal (60–90 V) or to increase the extent of in-source fragmentation of the $(M+Na)^{+}$ ion (150–170 V). Argon was present in the collision cell ($3.4–3.6 \times 10^{-5}$ mbar analyser Penning gauge reading) during both MS and MS/MS experiments. For MS/MS experiments, the collision energy was varied (40–60 V), depending on the resilience of the $(M+Na)^{+}$ ion, to achieve an optimal balance between parent and fragment ion intensities. A scan time of 2.4 or 2.5 s with an interscan delay of 0.1 s was used in all MS modes. Data were collected until an acceptable signal-to-noise ratio was achieved after the combination of individual spectra, typically 5–20. To provide additional mass accuracy for the analysis of MS/MS spectra, the calculated monoisotopic mass for the parent ion was used as a 'lock mass' to recalibrate these spectra after the combination of individual spectra.

3.3. Purification of enzymes

Recombinant plasmids pMKM1 derived from the wild-type strain of *R. marinus*[16] were used in this study. Culture of *Escherechia coli* DH5α used as a host strain for the plasmids was grown in Luria–Bertani (LB) medium with addition of 150 mg/mL of ampicillin at 37 °C for 7 h with shaking.[15] Recombinant laminaranase was purified according to the following scheme. All steps were carried out at 4 °C. Cells were separated by centrifugation (3000g, 30 min), washed twice with 10 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.02% NaN_3. Cells were re-suspended in a minimal volume of the same buffer containing 1 mM DTT, 0.2 M KCl, and 1 mg/mL of lysozyme (Sigma Chemical Co., USA). The mixture was then left at +4 °C for 45 min, sonicated (6×20 s) and centrifuged (30,000g, 20 min). The supernatant was adjusted to a pH value of 5.5 by addition of 2 N HCl, heated for 20 min at +60 °C and centrifuged (30,000g, 20 min). The resulting supernatant was dialysed against 20 mM AcONa buffer, pH 4.5, containing 5 mM $CaCl_2$ (buffer A). The crude β-1,3-1,4-glucanase preparation was loaded on a Toypearl SP-650M column (10×100 mm) equilibrated with buffer A. Bound protein was eluted with buffer A containing 0.25 M NaCl. Fractions containing laminaranase activity were pooled, concentrated to 15 mL on an Amicon PM-30 membrane, dialysed against 50 mM potassium phosphate buffer, pH 6.0 and loaded onto a Sephadex G-50 (Fine) column (22×960 mm) equilibrated with the same buffer. Fractions containing β-1,3-1,4-glucanase activity were pooled, dialyzed against deionized water and lyophilized.

Endo-β-1,3-glucanase (laminaranase) was isolated from the culture filtrate of *Oerskovia* sp. Following cultivation under conditions previously described,[30] cells were removed by centrifugation (3000g, 20 min). The supernatant was then simultaneously concentrated 20-fold and transferred to 20 mM Tris–HCl, pH 7.5 (buffer B) using of hollow fibers with a nominal molecular weight limit of 25 kDa ('Kirishi', Kirishi, Russia). The resulting protein solution was loaded onto a DEAE Sephadex A-50 column (100×50 mm) equilibrated with the same buffer. Bound protein was eluted with 1 M NaCl in buffer B. Fractions containing β-1,3-glucanase activity were pooled and the activity was precipitated by adding $(NH_4)_2SO_4$ to 1.7 M. The precipitate was separated by centrifugation (3000g, 20 min) and the supernatant was dialyzed sequentially against 20 mM AcONa, pH 4.0, and H_2O prior to lyophilization. The enzyme was obtained in 50% yield and had a specific β-1,3-D-glucanase activity of 120 U/mg.

Marine mollusc *S. sachalinensis* was kindly provided by Professor V.G. Tarasov (Institute of Bioorganic Chemistry, Far East Division of Russian Academy of Sciences) and then endo-β-1,3-glucanase was partially purified. Mollusc material was homogenised for 20 min at 4 °C in 3 vol of 20 mM AcONa buffer (pH 5.2), soluble fraction was removed by centrifugation (3000g for 20 min) and the supernatant was desalted on a Sephadex G-25 (fine) column equilibrated in the same buffer. Fractions with the laminaranase active enzyme (approx 100 mL) were pooled, applied onto a SP-Sephadex column, and eluted with 1 M NaCl in 20

mM AcONa buffer, pH 5.2. The active fraction was then used in further experiments.

Enzyme activity during the purification process was measured according to the Somogyi–Nelson method[23] using laminaran as a substrate.

3.4. Enzyme assays

β-1,3-1,4-D-Glucanase and β-1,3-D-glucanase (laminaranase) activities were determined by measuring the amount of reducing sugar released from laminaran. Standard assays for β-1,3-1,4-D-glucanase activity from *R. marinus* were carried out in 50 mM AcONa buffer, pH 6.0, (0.1 mL) at 75 °C for 10 min. One unit of the enzyme activity was defined as the amount of the enzyme required to produce 1 μmol of reducing sugar per minute from laminaran in the above conditions. β-Glucosidase activity was measured using *p*-nitrophenyl β-D-glucopyranoside as a substrate in 50 mM sodium phosphate buffer, pH 6.0, at 37 °C.[31] Other endoglycosidase activities were analysed by the Somogyi–Nelson method using appropriate polysaccharide substrates.

3.5. Mode of action of β-1,3-1,4-D-glucanase

The mode of action of the β-1,3-1,4-D-glucanase was investigated during the hydrolysis of $MeUmbG_{n=2-5}$ in 20 mM AcONa buffer, pH 4.0, at 37 °C, using substrate concentrations in the range from 0.8 to 1.2 mM over different time intervals and following the reaction by TLC. The reaction was terminated by freezing and lyophilisation. Analysis of the hydrolysis products was performed on a Waters Hypersil ODS column (200 × 4.8 mm) using linear gradient (0–90%) of MeCN in water with spectrophotometric detection at 254 nm.

3.6. Determination of kinetic characteristics of β-1,3-1,4-D-glucanase hydrolysis of MeUmb-β-laminaraoligosaccharides

The activity of the β-1,3-1,4-D-glucanase in the hydrolysis of $MeUmbG_2$, $MeUmbG_3$, and acetal-protected $MeUmbG_3$ was evaluated spectrofluorometrically after incubation of the reaction mixture in 50 mM AcONa buffer, pH 6.0, by measuring released MeUmb in accordance with Ref. 29. To reduce the rate of non-enzymatic hydrolysis of the substrate, the reaction was carried out at 37 °C.

The Michaelis–Menten parameters K_m and V_{max} were determined from the Lineweaver–Burk plots obtained by measuring the initial rate of hydrolysis of acetal-protected and non-protected MeUmb-β-laminaraoligosaccharides. The purified β-1,3-1,4-D-glucanase (0.009–0.010 U) was incubated with MeUmb-β-laminara-oligosaccharides in 50 mM AcONa buffer, pH 5.5 (0.1 mL). The Michaelis constant was determined for each substrate from the Michaelis–Menten equation by non-linear regression analysis.[32]

3.7. Investigation of transglycosylation activity of β-1,3-D-glucanases from *Oerskovia* and *S. sachalinensis*

The transglycosylation activity of β-1,3-D-glucanases toward laminaran and curdlan was investigated at concentrations from 5 to 100 mg/mL in 20 mM AcONa or sodium phosphate buffer over the pH range 3.5–8.5. Reaction products were analysed on a reverse-phase Waters Hypersil ODS column using UV detection at 254 nm. Product yields were calculated by integration of the corresponding chromatographic peaks.

3.8. Synthesis of 4-methylumbelliferyl β-laminaraoligosaccharides with d.p. 2–4

Curdlan (500 mg), MeUmbGlc*p* (100 mg) and 20–30 U of β-1,3-D-glucanase from *Oerskovia* were incubated in 20 mM AcONa buffer, pH 4.8, (5 mL) at 37 °C for 48 h. The extent of the reaction was tracked by TLC and reverse-phase HPLC. Products of the transglycosylation reaction were sequentially isolated on a Bio-Gel P2 extra fine (Bio Rad, USA) column (10 × 1200 mm, flow rate 9 mL/h) in H_2O followed by an INERTSIL PREP-ODS column (20 × 250 mm) using a linear gradient (0–100%) of MeCN in water.

3.9. 4-Methylumbelliferyl β-laminarabioside

$[\alpha]_D$ −60.3° (*c* 0.335, water).

3.10. 4-Methylumbelliferyl β-laminaratrioside

$[\alpha]_D$ −44.2° (*c* 0.335, water).

3.11. 4-Methylumbelliferyl β-laminaratetraoside

$[\alpha]_D$ −26.3° (*c* 0.335, water).

3.12. Synthesis of 4-methylumbelliferyl β-laminaraoligosaccharides with d.p 5 and 6

Laminaran (200 mg), MeUmbGlc*p* (100 mg) and mollusc β-1,3-D-glucanase (20 U) were incubated in 50 mM AcONa buffer, pH 4.8, (5 mL) for 48 h at 37 °C. Products of the reaction were isolated as described above for the di-, tri-, and tetrasaccharide analogues.

3.13. 4-Methylumbelliferyl β-laminarapentaoside

$[\alpha]_D$ −21.0° (*c* 0.335, water).

3.14. 4-Methylumbelliferyl β-laminarahexaoside

$[\alpha]_D$ −18.4° (*c* 0.335, water).

3.15. Synthesis of 4-methylumbelliferyl ω-4,6-*O*-benzylidene-β-laminaraoligosaccharides with d.p 3–5

Derivatisation of $MeUmbG_3$, $MeUmbG_4$, and $MeUmbG_5$ was performed as follows. A mixture of 3 mmol $MeUmbG_n$, 10 mmol (1.5 mL) of α,α-dimethoxytoluene, and 50 mg of *p*-toluenesulfonic acid monohydrate in dry *N*,*N*-dimethylformamide (10 mL) was incubated overnight at 37 °C.[33] The resulting syrup was fractionated on an INERTSIL PREP-ODS column using a linear gradient (0–100%) of MeCN in water.

3.16. 4-Methylumbelliferyl ω-4,6-*O*-benzylidene-β-laminaratrioside

NMR ^{1}H δ: 5.283 (1 H, $J_{H1,H2}$ 7.93 Hz, H-1-1), 4.923 (1 H, $J_{H1,H2}$ 7.86 Hz, H-1-3), 4.868 (1 H, $J_{H1,H2}$ 7.86 Hz, H-1-2), 4.384 (1 H, $J_{H5,H6a}$ 4.85, $J_{H6a,H6b}$ 10.5 Hz, H-6a-3), 3.982 (1 H, $J_{H5,H6a}$ 2.36, $J_{H6a,H6b}$ 12.4 Hz, H-6a-1), 3.976 (1 H, $J_{H5,H6a}$ 1.8, $J_{H6a,H6b}$ 11.9 Hz, H-6a-2), 3.934 (1 H, $J_{H2,H3}$ 9.2, $J_{H3,H4}$ 8.8 Hz, H-3-1), 3.892 (1 H, $J_{H5,H6b}$ 9.8 Hz, H-6b-3), 3.838 (1 H, H-2-1), 3.824 (1 H, $J_{H2,H3}$ 9.2, $J_{H3,H4}$ 8.9 Hz, H-3-3), 3.818 (1 H, $J_{H5,H6b}$ 5.5 Hz, H-6b-1), 3.814 (1 H, $J_{H3,H4}$ 8.9, $J_{H4,H5}$ 9.5 Hz, H-4-2), 3.765 (1 H, $J_{H4,H5}$ 9.5 Hz, H-5-1), 3.743 (1 H, $J_{H5,H6b}$ 4.8 Hz, H-6b-2), 3.684 (1 H, $J_{H4,H5}$ 9.2 Hz, H-4-3), 3.659 (1 H, H-4-1), 3.640 (1 H, $J_{H2,H3}$ 9.3 Hz, H-3-2), 3.633 (1 H, H-5-3), 3.632 (1 H, H-2-2), 3.557 (1 H, H-5-2), 3.521 (1 H, H-2-3). $[\alpha]_D$ −23.2° (*c* 0.15, water).

3.17. 4-Methylumbelliferyl ω-4,6-*O*-benzylidine-β-laminaratetraoside

$[\alpha]_D$ −14.2° (*c* 0.15, water).

3.18. 4-Methylumbelliferyl ω-4,6-*O*-benzylidene-β-laminarapentaoside

$[\alpha]_D$ −9.5° (*c* 0.15, water).

3.19. Synthesis of 4-methylumbelliferyl ω-4,6-*O*-ethylidine-β-laminarabioside

Synthesis of 4,6-*O*-ethylidene-modified MeUmb β-laminarabioside was performed as described above for a benzylidene-protected analogue.

3.20. 4-Methylumbelliferyl ω-4,6-*O*-ethylidine-β-laminarabioside

$[\alpha]_D$ −21.4° (*c* 0.15, water).

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