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A lichenase-like family 12 *endo*-(1→4)-β-glucanase from *Aspergillus japonicus*: study of the substrate specificity and mode of action on β-glucans in comparison with other glycoside hydrolases

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Abstract—Using anion-exchange chromatography on Source 15Q followed by hydrophobic interaction chromatography on Source 15 Isopropyl, a lichenase-like *endo*-(1→4)-β-glucanase (BG, 28 kDa, pI 4.1) was isolated from a culture filtrate of *Aspergillus japonicus*. The enzyme was highly active against barley β-glucan and lichenan (263 and 267 U/mg protein) and had much lower activity toward carboxymethylcellulose (3.9 U/mg). The mode of action of the BG on barley β-glucan and lichenan was studied in comparison with that of *Bacillus subtilis* lichenase and *endo*-(1→4)-β-glucanases (EG I, II, and III) of *Trichoderma reesei*. The BG behaved very similar to the bacterial lichenase, except the tri- and tetrasaccharides formed as the end products of β-glucan hydrolysis with the BG contained the β-(1→3)-glucoside linkage at the non-reducing end, while the lichenase-derived oligosaccharides had the β-(1→3)-linkage at the reducing end. The BG was characterized by a high amino acid sequence identity to the EG of *Aspergillus kawachii* (UniProt entry Q12679) from a family 12 of glycoside hydrolases (96% in 162 identified aa residues out of total 223 residues) and also showed lower sequence similarity to the EglA of *Aspergillus niger* (O74705).

Keywords: *Aspergillus japonicus*; *Bacillus subtilis*; Barley β-glucan; *endo*-(1→4)-β-Glucanase; Lichenan; Lichenase; *Trichoderma reesei*

1. Introduction

β-(1→3)-(1→4)-Glucans are polysaccharides found in the cell walls of cereals. They play the role of storage polysaccharides and are most abundant in the endosperm of barley, oat, rye, rice, sorghum, and wheat grain.[1] A similar type of β-glucan (called lichenan), containing mixed β-(1→3)- and β-(1→4)-glucoside linkages, has been found in lichens. Lichenan from *Cetraria islandica* has a regular structure and consists mostly of cellotriosyl units linked by a single β-(1→3)-glucoside bond (the ratio between β-(1→3)- and β-(1→4)-linkages is 1:2).[2] About 90% of barley β-glucan consists of cellotriosyl and cellotetraosyl residues, which are also linked by a single β-(1→3)-glucoside bond (the ratio between β-(1→3)- and β-(1→4)-linkages is 1:2.3–2.5). The degree of polymerization is about 1200, depending on the method of isolation of the polysaccharide.[1,3]

The biodegradation of β-(1→3)-(1→4)-glucans in nature is catalyzed by glycoside hydrolases differing in the substrate specificity (for simplicity, in the subsequent text the terms 'β-glucan' and 'β-glucanase' will mean β-(1→3)-(1→4)-glucan and the enzyme possessing an activity against this polysaccharide). Cellulases (*endo*-(1→4)-β-glucanases, EC 3.2.1.4), belonging to different families of glycoside hydrolases,[4,5] are able to split internal β-(1→4)-linkages in β-glucans, thus displaying β-glucanase activity[6–8] (see classification into families

Abbreviations: BG, β-glucanase; BGL, β-glucosidase; CMC, carboxymethylcellulose; EG, *endo*-(1→4)-β-glucanase; GPC, gel-permeation chromatography; MS, mass spectrometry; MWD, molecular weight distribution; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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also at http://afmb.cnrs-mrs.fr/CAZY/). Licheninases (lichenases, (1→3)-(1→4)-β-D-glucan 4-glucanohydrolases, EC 3.2.1.73), the enzymes acting specifically on β-glucans, strictly hydrolyze β-(1→4)-linkages (like cellulases), but they typically have no activity against true β-(1→4)-glucans (such as cellulose and carboxymethylcellulose, CMC).[6,9,10] Most of the known lichenases are of bacterial origin and belong to family 16 of glycoside hydrolases.[6] The most studied lichenases are produced by *Bacillus* species.[11–17] Plant enzymes with similar specificity are grouped into family 17.[10] Quite recently (1→3)-(1→4)-β-glucanases have been found in fungi. However, the number of fungal lichenase-like enzymes known to date are rather low, and information on their properties is scarce. The first fungal gene encoding lichenase (from *Orpinomyces* sp.) was identified in 1997.[18] Later, enzymes from *Cochliobolus carbonum*[19,20] and *Talaromyces emersonii*[21] have been described.

Amongst other types of enzymes having the β-glucanase activity, laminarinases (*endo*-(1→3)-β-glucanases, EC 3.2.1.39, and *endo*-(1→3)(4)-β-glucanases, EC 3.2.1.6) as well as β-glucosidases (EC 3.2.1.21) should be mentioned.[6,10,22] At least, two fungal *endo*-(1→3)(4)-β-glucanases (from *C. carbonum*[19] and *Phaffia rhodozyma*[23]) have been described. β-Glucosidases from family 3 remove glucosyl residues from the non-reducing ends of β-glucan and oligosaccharide molecules and typically have wide substrate specificity hydrolyzing β-(1→3), β-(1→4) and other types of glycoside linkages.[10,22]

Although carbohydrases and other enzymes produced by black *Aspergilli* have been extensively studied,[24,25] the presence of specific β-glucanases (lichenases) has not been clearly demonstrated in these fungi, except for the β-glucanase gene fragment (translated amino acid sequence, UniProt entry Q9HGU1, belonging to family 16 of glycoside hydrolases).

In this paper, we report the properties of a lichenase-like β-glucanase (BG) from *Aspergillus japonicus*, which is highly homologous to *Aspergillus kawachii endo*-(1→4)-β-glucanase from family 12 (UniProt entry Q12679). The enzyme has extremely high activity against barley β-glucan and lichenan, while its cellulase (CMCase) activity is relatively low (about 70 times lower than the β-glucanase activity). The mode of action of the *Aspergillus japonicus* BG on β-glucans was studied in comparison with that of *Bacillus subtilis* lichenase and three *endo*-(1→4)-β-glucanases from *Trichoderma reesei* (*Hypocrea jecorina*).

2. Results

2.1. β-Glucanase purification

The crude *A. japonicus* preparation possessed a high activity toward barley β-glucan together with the 28-fold lower CMCase activity. Other types of activities (against xylan, xyloglucan, polygalacturonic acid, starch, and other polysaccharides) were also present. The first step of purification included anion-exchange chromatography on a Source 15Q column. Enzyme activities toward CMC and barley β-glucan were analyzed in protein fractions obtained as a result of NaCl gradient elution. The protein fraction displaying the highest BG activity, which was eluted at 0.04 M of NaCl, was subjected to further purification by hydrophobic interaction chromatography on a Source 15 Isopropyl column.

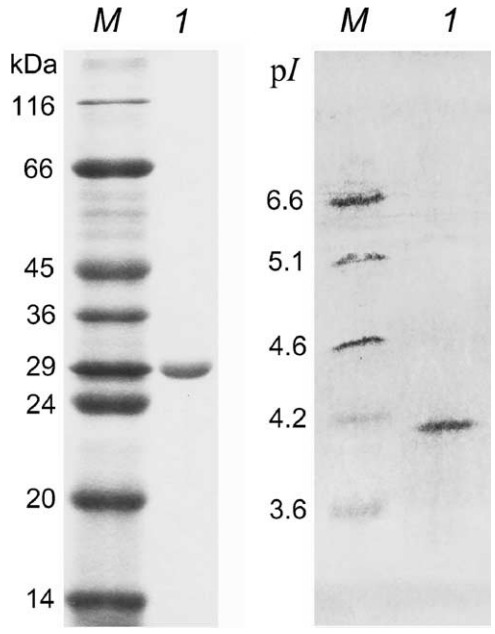

Figure 1. SDS-PAGE and isoelectrofocusing of purified BG from *A. japonicus*. Lanes: *M*, protein markers with different molecular masses and pI; (1) BG.

The enzyme, highly active toward barley β-glucan and lichenan, was obtained as a result of purification (its substrate specificity is discussed below). It was homogeneous according to SDS-PAGE and isoelectrofocusing data (Fig. 1). The molecular mass of the BG was found to be 28 kDa, pI 4.1. The final yield was 19 mg of protein (4.8% of the initial protein in the crude multienzyme sample). The yield by the BG activity was 26%.

2.2. Identification of peptides in β-glucanase using mass spectrometry

A piece of a protein band from the SDS-PAGE gel that corresponded to the *A. japonicus* BG was digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry (MS).[26] The corresponding mass spectrum is shown in Figure 2. Then, the MASCOT program search (http://www.matrixscience.com) was carried out to analyze the peptide masses that were obtained against known glycoside hydrolases in the NCBI and SWISS-PROT databases.

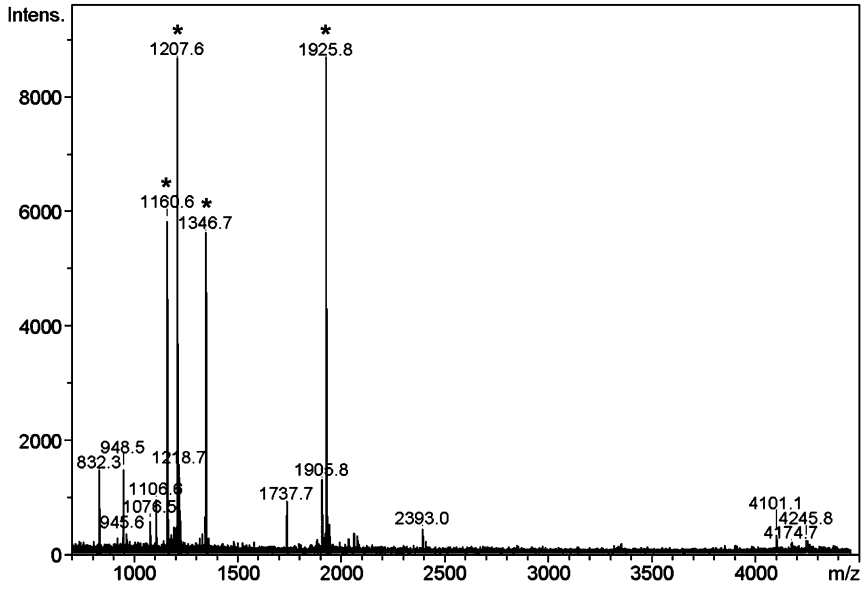

Figure 2. MALDI-TOF mass spectrum of peptides derived from the in-gel tryptic digest of *A. japonicus* BG. Peptides subjected to further analysis by MS/MS are marked with asterisks.

The MASCOT search found six matching tryptic peptides in the amino acid sequence of the *endo*-(1→4)-β-glucanase (EG) from *A. kawachii* (SWISS-PROT/UniProt entry Q12679)[27] belonging to family 12 of glycoside hydrolases (see Table 1). The same sequence is affiliated to the EG from *A. niger* in the NCBI database (S55931). The matching peptides covered 21% of the sequence. Typically, a protein is generally considered identified by MALDI-TOF MS with sufficient confidence when at least five peptide masses are matched and the sequence coverage is not less than 15%.[28] More detailed search against amino acid sequence of the *A. kawachii* EG and sequences of other known family 12 *Aspergilli* enzymes, using ExPASy mass spectrometry tools (FindPept and FindMod, http://au.expasy.org/tools/), revealed the presence of three additional peptides in the *A. japonicus* BG (Table 1). The first of them (*m*/*z* 1905.8) matched the peptide 176–193 in the EG of *A. kawachii* (Q12679) but with a single W→Y amino acid substitution. The second peptide (*m*/*z* 4101.1) matched the peptide 103–139 from another family 12 enzyme (*A. niger* EglA, SWISS-PROT/UniProt entry O74705).[29] Two forms of the third peptide (with *m*/*z* 4174.7 and 4245.8) represented a modified N-terminal peptide of both the mature EG of *A. kawachii* and EglA of *A. niger* (without signal peptides). Any other matches in masses

Table 1. Identification of peptides in the *A. japonicus* β-glucanase using MALDI-TOF MS and MS/MS

m/*z* observed	*m*/*z* calculated	Peptide sequence identified[a]	Method of identification	UniProt entry	Residues
945.5	945.53	QIATATVGGK	MASCOT	Q12679	149–158
948.5	948.51	YGSVQPIGK	MASCOT	Q12679	140–148
1160.6	1160.56	**LSSSGASWHTK**	MASCOT+MS/MS	Q12679	54–64
1207.6	1207.57	**WTWSGGEGTVK**	MASCOT+MS/MS	Q12679	65–75
1218.7	1218.56	SYSNSGLTFDK	MASCOT	Q12679	76–86
1346.7	1346.65	**SYSNSGLTFDKK** (1 MC)	MASCOT+MS/MS	Q12679	76–87
1905.8	1905.92	TYSFVAGSPINS<u>Y</u>SGDIK	ExPASY FindMod	Q12679	176–193
1925.8	1925.90	**SWEVWYGTS<u>V</u>QAGAEQK**	MS/MS	Q12679	159–175
4101.1	4100.89	QDNTNVNADVAYDLFTAANVDHATSSGDYELMIWLAR	ExPASY FindPept	O74705	103–139
4174.7	4174.77	QTMCSQYDSASSPPYSVNQNLWGEYQGTGSQCVYVDK[b]	ExPASY FindMod	Q12679 O74705	17–53
4245.8	4245.81	QTMCSQYDSASSPPYSVNQNLWGEYQGTGSQCVYVDK[b]	ExPASY FindMod	Q12679 O74705	17–53

[a] Peptides, whose structures were confirmed by de novo sequencing of MS/MS spectra, are shown in bold. Single amino acid substitutions in peptides are underlined. MC denotes missed cleavage by trypsin. Since the MS/MS cannot distinguish between Leu and Ile residues (they have the same masses), Ile may be in the place of Leu in the appropriate positions of the identified peptides.

[b] Peptides with *m*/*z* of 4174.7 and 4245.8 represent the same N-terminal peptide of both *A. kawachii* EG and *A. niger* EglA (mature proteins without signal peptides), where one and two Cys residues, respectively, were modified by free acrylamide in the SDS-PAGE gel and additionally the N-terminal Gln residue was modified to pyroglutamic acid.

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                                                                    *
Q12679   1 mklsmtlslfaatamgQTMCSQYDSASSPPYSVNQNLWGEYQGTGSQCVYVDKLSSSGAS 60
O74705   1 mklpvslamlaatamgQTMCSQYDSASSPPYSVNQNLWGEYQGTGSQCVYVDKLSSSGAS 60

               v          *          **              v
Q12679  61 WHTKWTWSGGEGTVKSYSNSGLTFDKKLVSDVSSIPTSVTWSQDDTNVQADVSYDLFTAA 120
O74705  61 WHTEWTWSGGEGTVKSYSNSGVTFNKKLVSDVSSIPTSVEWKQDNTNVNADVAYDLFTAA 120

                          *         *         *                 *
Q12679 121 NADHATSSGDYELMIWLARYGSVQPIGKQIATATVGGKSWEVWYGTSTQAGAEQKTYSFV 180
O74705 121 NVDHATSSGDYELMIWLARYGNIQPIGKQIATATVGGKSWEVWYGSTTQAGAEQRTYSFV 180

                        v
Q12679 181 AGSPINSWSGDIKDFFNYLTQNQGFPASSQHLITLQCGTEPFTGGPATFTVDNWTASVN 239
O74705 181 SESPINSYSGDINAFFSYLTQNQGFPASSQYLINLQFGTEAFTGGPATFTVDNWTASVN 239
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Figure 3. Alignment of amino acid sequences of *A. kawachii* EG (UniProt entry Q12679) and *A. niger* EglA (O74705). Signal peptides are shown in small letters. Conserved residues are shown in bold. Potential sites of cleavage with trypsin are marked with '*' or 'v'. Peptides from the *A. japonicus* BG matching the sequences of the *A. kawachii* EG and *A. niger* EglA are underlined.

between tryptic peptides from the *A. japonicus* BG and those from other known *Aspergilli* enzymes listed in the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/) were not found. It should be noted that the *A. kawachii* EG (Q12679) and *A. niger* EglA (O74705) share 87% of sequence identity. The alignment of their sequences is shown in Figure 3.

In order to verify the sequences of peptides from the *A. japonicus* BG, four of them (with *m*/*z* 1160.6, 1207.6, 1346.7, 1925.8) were analyzed by tandem mass spectrometry (MS/MS).[26] De novo sequencing of the peptides was carried out using the data from fragmentation mass spectra. The sequences of the first three peptides found by the MASCOT search were completely confirmed by the data of MS/MS (Table 1). Additionally, the complete sequence of the peptide with *m*/*z* 1925.8 was determined. This sequence was almost the same as that of peptide 159–175 from the *A. kawachii* EG (Q12679), except for one residue (Val instead of Thr). Because of this substitution (2 Da difference), this peptide was not recognized by the MASCOT program.

As a summary of this section, peptides from the *A. japonicus* BG matching the sequences of the *A. kawachii* EG (Q12679) and *A. niger* EglA (O74705) are underlined in Figure 3. The higher homology was observed to the *A. kawachii* EG. Its overall sequence coverage with peptides identified in the *A. japonicus* BG became 70% (96% sequence identity in the regions underlined in Figure 3). Taking into account the fact that the theoretical C-terminal tryptic peptides of both *A. kawachii* EG and *A. niger* EglA have a rather conserved sequence (the same should be valid for *A. japonicus* BG), but have masses too high (*m*/*z* >5000) to be visible in the mass spectrum, one may expect the overall sequence identity between the *A. japonicus* BG and *A. kawachii* EG to be not less than 90–95%.

2.3. Enzyme substrate specificity

Specific activities of the purified *A. japonicus* BG toward different substrates and the enzyme's major properties are given in Table 2. Substrate specificity and properties of other glycoside hydrolases, which were taken for a comparison study (see the subsequent section), are also present in Table 2. The *A. japonicus* BG displayed the highest activity against barley β-glucan and lichenan

Table 2. Basic properties and specific activities of the purified enzymes (pH 5.0, 50 °C)

Property	*A. japonicus* BG	*B. subtilis* lichenase	*T. reesei*			
			EG I	EG II	EG III	BGL1
Mol. mass (kDa)	28	27	56	51	23	74
pI	4.1	8.1; 8.4	4.9	5.9	7.4	8.9
pH-optimum	4.7	6.6	5.0	4.5	5.7	4.8
pH_{50}[a]	3.3–5.9	4.9–8.6	3.5–6.6	2.4–6.6	3.9–8.3	3.9–6.0
Activity (U/mg) toward						
Barley β-glucan	263	176	33	50	17	23
Lichenan	267	200	33	52	24	52
CMC	3.9	0	52	49	20	<0.5
Laminarin	0	0	0	0	0	45
Xyloglucan	0	0	64	0	20	0
Avicel	0	0	0.20	0.17	0.06	0
K_m for β-glucan (mg/mL)	3.3 ± 0.6	0.84 ± 0.16	1.18 ± 0.24	1.13 ± 0.21	1.80 ± 0.18	5.6 ± 1.0
k_{cat} for β-glucan (s^{-1})	162 ± 9	98 ± 6	38 ± 2	52 ± 4	11 ± 1	56 ± 5

[a] pH region, where the enzyme retains 50% or more of the maximum activity.

(polymers with mixed β-(1→3)-, β-(1→4)-glucoside linkages). Activity toward CMC (modified β-(1→4)-glucan) was ~70 times lower. Activity toward laminarin (β-(1→3)-glucan) was absent, indicating that the enzyme has a strict specificity toward β-(1→4)-linkages between D-Glc*p* residues. The enzyme had no activities against Avicel cellulose, xyloglucan from tamarind, birch xylan, galactomannan, linear, and branched arabinans, galactan, polygalacturonic acid, starch, and *p*-nitrophenyl glycosides of β-D-Glc*p*, β-cellobiose, β-lactose, β-D-Xyl*p*, α-D-Xyl*p*, α-L-Ara*f*, α-L-Ara*p*.

Such substrate specificity of the *A. japonicus* BG (extremely high activity against barley β-glucan and lichenan) resembles that of bacterial lichenases (EC 3.2.1.73) rather than cellulases (*endo*-(1→4)-β-glucanases, EC 3.2.1.4). True *T. reesei* cellulases (EG I, II, and III) had much lower specific activities against barley β-glucan and lichenan (Table 2), the ratio of β-glucanase to CMCase activity for them being varied in the range of 0.63–1.0. At the same time, true lichenases (such as that from *B. subtilis*, Table 2) have no activity against CMC at all. So, the *A. japonicus* enzyme seems to be a non-typical *endo*-(1→4)-β-glucanase (EC 3.2.1.4) with enhanced β-glucanase (lichenase) activity.

Regarding other important properties of the *A. japonicus* BG, the enzyme had its activity optimum at pH 4.7, and its Michaelis constant (K_m) in hydrolysis of barley β-glucan was found to be 3.3 mg/mL. The value of K_m was higher than the corresponding parameters for *B. subtilis* lichenase and endoglucanases from *T. reesei* (Table 2). However, the value of the catalytic constant, k_{cat}, was also higher in the case of BG.

2.4. Enzyme mode of action

In order to reveal the mode of action of the isolated β-glucanase (*endo*- or *exo*-type), we studied its action on barley β-glucan. Aliquots of the reaction mixture were taken at different times of hydrolysis, and they were analyzed for reducing sugars and also for molecular weight distribution (MWD) of the hydrolysis products by gel-permeation chromatography (GPC). Hydrolysis was carried out at 40 °C and pH 5.0 for 24 h, although the most characteristic changes happened in the first 1 h of the reaction. The changes in the elution profiles during the GPC of β-glucan at different times of hydrolysis by *A. japonicus* BG revealed its *endo*-depolymerase mode of action (Fig. 4A). As seen from the figure, the initial peak of β-glucan (initial $M_r \sim 200$ kDa) was quickly widened in the course of reaction, its M_r decreased (it migrated to the right side on the chromatogram), but at the same time the formation of low-molecular-weight products was not observed at the initial stage of hydrolysis (curves 2–4). Such products, corresponding to short oligomers, appeared only by the end of the reaction (curves 5 and 6).

Similar (*endo*-depolymerase) patterns of changes in the β-glucan elution profiles were observed for the *B. subtilis* lichenase as well as for EG I (Cel7B), EG II (Cel5A), and EG III (Cel12A) from *T. reesei* (data are shown only for lichenase, Fig. 4B). These enzymes (plus β-glucosidase BGL1, that is, Cel3A of *T. reesei*) were taken for a comparison in our study. As expected, the BGL1 behaved like a true *exo*-acting enzyme in hydrolysis of β-glucan. For this enzyme, the height of the substrate peak decreased, and this was accompanied by the formation of low-molecular product (glucose) even in the initial period of the reaction; however, the formation of products with middle-range molecular weights was not observed (Fig. 4C).

In Figure 5 the dependencies of average molecular weight (M_r) of the polymeric β-glucan upon the degree of hydrolysis (% of hydrolyzed glycoside bonds calculated from the reducing-sugar assay) are shown. For

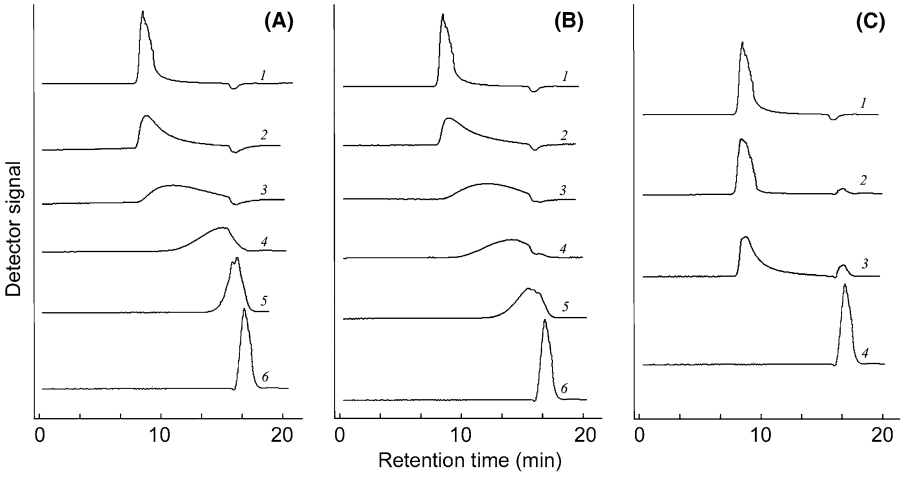

Figure 4. Analysis of β-glucan hydrolysis products using GPC on TSK G3000SWXL column. (A), *A. japonicus* BG; (B), *B. subtilis* lichenase; (C) *T. reesei* BGL1. Hydrolysis time for (A) and (B): (1) 0 min; (2) 10 min; (3) 20 min; (4) 40 min; (5) 6 h; (6) 24 h. Hydrolysis time for (C): (1) 0 min; (2) 2 h; (3) 6 h; (4) 48 h.

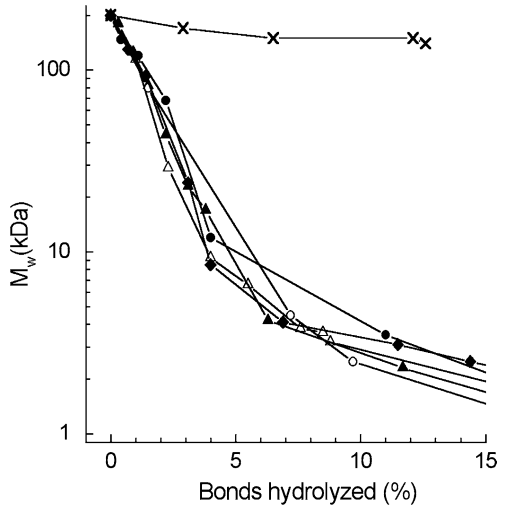

Figure 5. Changes in the average molecular weight (M_r) of polymeric β-glucan during enzymatic hydrolysis: (×) *T. reesei* BGL1; (▲) *A. japonicus* BG; (△) *B. subtilis* lichenase; (●) *T. reesei* EG I; (○) *T. reesei* EG II; (♦) *T. reesei* EG III.

all endo-acting enzymes (lichenase, BG, EG I, EG II, EG III), the M_r of β-glucan decreased rapidly even at the initial stage of the reaction. On the contrary, in the case of the *exo*-acting BGL1 the average M_r of β-glucan decreased very slowly in spite of the growing degree of hydrolysis.

The end aliquots, obtained after the exhaustive hydrolysis of barley β-glucan by the above-mentioned enzymes, were analyzed by HPLC using a column containing silica with bonded amino phase (Fig. 6). Only two major products with similar retention times were found in the case of *A. japonicus* BG and *B. subtilis* lichenase (oligosaccharides designated as A and B; A′ and B′ on chromatograms 1 and 2). These oligosaccharides were different from cellotriose and cellotetraose as a comparison with chromatographic standards shows (upper chromatogram in Fig. 6). It has been reported[6] that, in the hydrolysis of barley β-glucan and lichenan, bacterial lichenases form oligosaccharides containing β-(1→3)-glucoside linkages at the reducing ends. This happens because the lichenases split a β-(1→4)-linkage strictly after the β-(1→3)-linkage. So, the products A and B seem to have the following structure:

A : β-Glc-(1→4)-β-Glc-(1→3)-β-Glc

B : β-Glc-(1→4)-β-Glc-(1→4)-β-Glc-(1→3)-β-Glc

The end products of β-glucan hydrolysis obtained under the action of *T. reesei* endoglucanases were different (Fig. 6, chromatograms 3–5). The EG I formed tetrasaccharide, similar by the retention time to oligosaccharide B (B′), together with large amounts of glucose and cellobiose. The EG II formed mainly trisaccharide, similar by the retention time to oligosaccharide A (A′), together

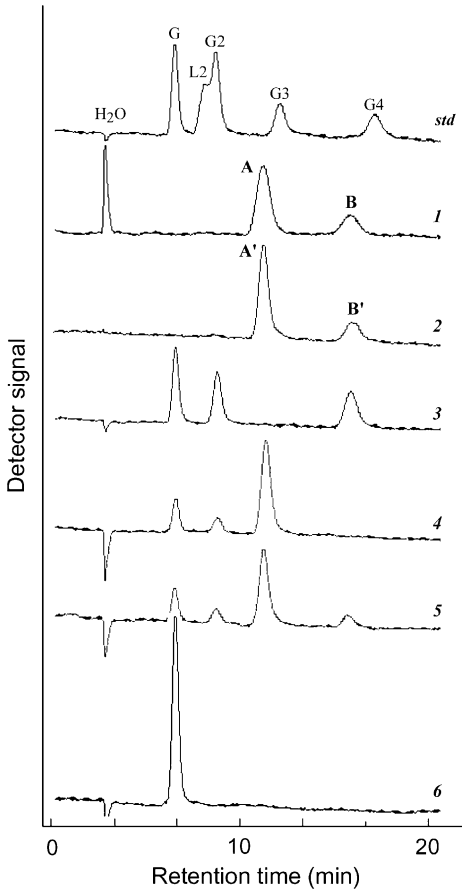

Figure 6. HPLC analysis of the end products of β-glucan hydrolysis using a silica column with bonded amino phase and 7:3 CH_3CN–H_2O as a mobile phase at a flow rate of 1 mL/min. (1) *B. subtilis* lichenase; (2) *A. japonicus* BG; (3–6), *T. reesei* EG I, EG II; EG III, BGL1, respectively. The upper chromatogram represents chromatographic standards: glucose (G), laminaribiose (L2), cellobiose (G2), cellotriose (G3), cellotetraose (G4).

with lesser amounts of glucose and cellobiose; tetrasaccharide B (B′) was completely absent. The major product for the EG III was trisaccharide A (A′), together with glucose, cellobiose, and tetrasaccharide B (B′). Previously, we have observed similar patterns of barley β-glucan hydrolysis products (glucose, cellobiose, tri- and/or tetrasaccharide) for five different purified *endo*-(1→4)-β-glucanases from *Chrysosporium lucknowense*.[8] As expected, the only product of the BGL1 action on β-glucan was glucose (chromatogram 6).

It should be noted that the formation of glucose and cellobiose in reactions catalyzed by *T. reesei* endoglucanases cannot be explained by the enzyme contamination with *exo*-hydrolases or β-glucosidases since the enzymes were homogeneous according to the SDS-PAGE and isoelectrofocusing data, and they did not display the activity toward *p*-nitrophenyl β-D-glucoside.[37] The activity against *p*-nitrophenyl derivatives of β-cellobiose, β-lactose, β-D-Xyl*p*, α-D-Xyl*p*, α-L-Ara*f*, α-L-Ara*p* was also absent, except for the EG I (Cel7A) possessing

the activity against *p*-nitrophenyl β-cellobioside and β-lactoside,[37] which is a property inherent to this enzyme and other cellulases from family 7.[38,43]

In order to find out whether the oligosaccharides A and A′ (B and B′) formed under the action of *A. japonicus* BG and *B. subtilis* lichenase are the same or not, they were subjected to sequential hydrolysis with the BGL1. Taking into account the fact that barley β-glucan is composed of cellotriosyl and cellotetraosyl residues linked by a single β-(1→3)-glucoside bond,[1] possible structures of trisaccharides (A and A′) and tetrasaccharides (B, B′, and B″), which may be formed in the enzymatic hydrolysis and are different from cellotriose and cellotetraose, are shown in Figure 7. As already mentioned above, the products obtained from β-glucan under the action of *B. subtilis* lichenase should have the structures of oligosaccharides A and B. Since the BGL1 acts on glycoside molecules from the non-reducing end, laminaribiose (L2) should be formed as an intermediate product in the BGL1-catalyzed hydrolysis of A and B. If the products were A′ and B′, then the BGL1-catalyzed hydrolysis would give cellobiose (G2) and cellotriose (G3) as intermediate products. The third possible tetrasaccharide (B″) would give the trisaccharide A′ (but not cellotriose) and cellobiose as intermediate products.

The real chromatograms of saccharides formed during the BGL1-catalyzed hydrolysis of oligosaccharides (end products of β-glucan hydrolysis with lichenase and BG) are shown in Figures 8 and 9. The formation of laminaribiose was observed in the case of lichenase-derived oligosaccharides A and B (Fig. 8), confirming their structures shown above and the lichenase mode of action on β-glucan in general. However, in the case

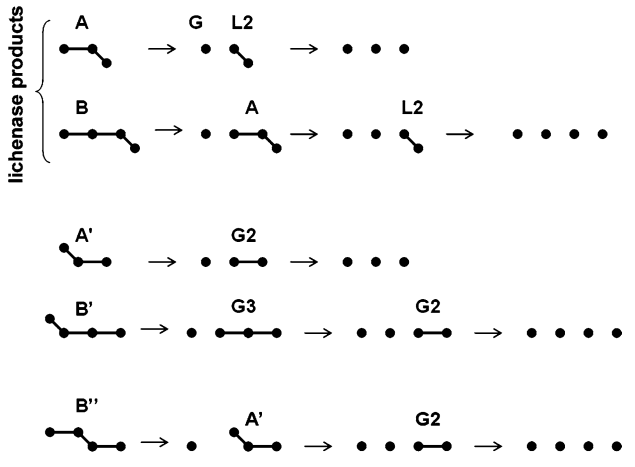

Figure 7. Digestion of theoretical end products of β-glucan hydrolysis containing mixed β-(1→4)- and β-(1→3)-glucoside linkages (trisaccharides A and A′; tetrasaccharides B, B′, and B″) with BGL1. Non-reducing ends are located on the left of oligosaccharide molecules. (●) denotes the β-D-Glc*p* residue; (–) β-(1→4)-linkage; (\) β-(1→3)-linkage; G, glucose; L2, laminaribiose; G2, cellobiose; G3, cellotriose.

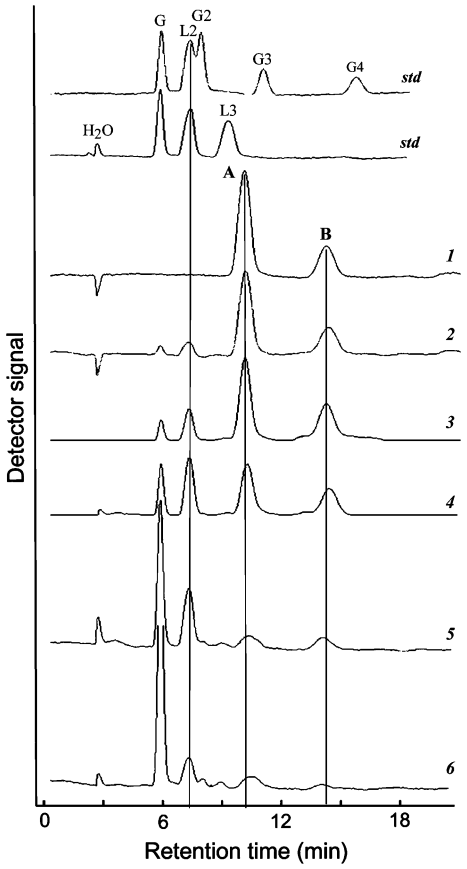

Figure 8. HPLC analysis of saccharides formed in the reaction system containing the BGL1 of *T. reesei* and the end products of β-glucan hydrolysis obtained under the action of *B. subtilis* lichenase. Reaction time: (1) 0 h; (2) 1 h; (3) 3 h; (4) 6.5 h; (5) 24 h; (6) 48 h. The upper chromatograms represent chromatographic standards: glucose (G), laminaribiose (L2), cellobiose (G2), cellotriose (G3), cellotetraose (G4), laminaritriose (L3).

of BG-derived oligosaccharides the formation of laminaribiose was not detected. Instead, cellobiose and cellotriose were formed as intermediate products (Fig. 9), indicating that the end products of β-glucan hydrolysis obtained under the action of *A. japonicus* BG have the structure of oligosaccharides A′ and B′ in Figure 7, that is, they are different from those obtained in β-glucan hydrolysis by *B. subtilis* lichenase. In other words, when acting on β-glucan, the *A. japonicus* BG splits the β-(1→4)-glucoside linkage *before* the β-(1→3)-linkage (if to move from the non-reducing end to the reducing end of the polymer molecule), whereas the *B. subtilis* lichenase acts on the β-(1→4)-glucoside linkage *after* the β-(1→3)-linkage (Fig. 10).

Such modes of action of the *A. japonicus* BG and *B. subtilis* lichenase were also confirmed on lichenan as a substrate (mixed β-(1→3)-(1→4)-glucan of simpler structure than barley β-glucan; it consists of cellotriosyl units linked by a single β-(1→3)-glucoside bond[2]). As expected, oligosaccharide A was formed as a major product in lichenan hydrolysis by lichenase, while oligo-

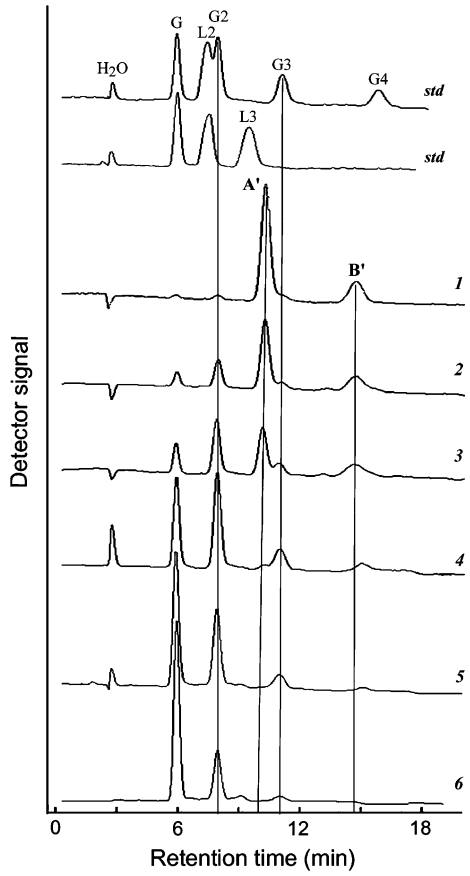

Figure 9. HPLC analysis of saccharides formed in the reaction system containing the BGL1 of *T. reesei* and the end products of β-glucan hydrolysis obtained under the action of *A. japonicus* BG. Reaction time: (1) 0 h; (2) 3 h; (3) 6.5 h; (4) 24 h; (5) 48 h; (6) 96 h. The upper chromatograms represent the same chromatographic standards as those in Figure 8.

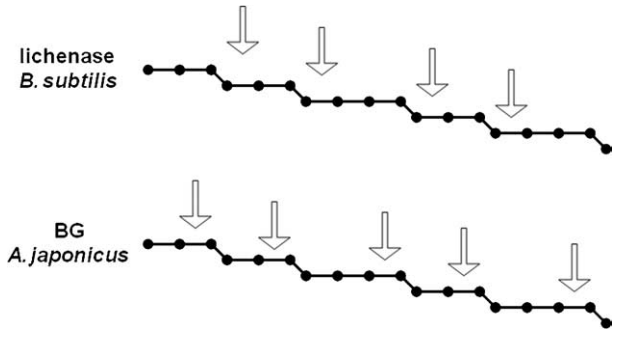

Figure 10. Mode of action of *B. subtilis* lichenase and *A. japonicus* BG on barley β-glucan. Non-reducing ends are located on the left of polymer molecules. (●) denotes the β-D-Glc*p* residue; (–) β-(1→4)-linkage; (\) β-(1→3)-linkage.

saccharide A′ was the end product in the case of BG (data are not shown). When these products were subjected to sequential hydrolysis with the BGL1, laminaribiose, and cellobiose, respectively, were formed as intermediate products.

3. Discussion

The enzyme highly active on mixed β-(1→3)-(1→4)-glucans (barley β-glucan and lichenan) was isolated from the *A. japonicus* culture filtrate. Its specific activity against CMC was much lower (~70-fold) than the β-glucanase activity (Table 2). The enzyme was not active toward laminarin (β-(1→3)-glucan) indicating that it has a strict specificity toward β-(1→4)-glucoside linkages. Such substrate specificity resembles that of bacterial lichenases (EC 3.2.1.73) belonging to family 16 of glycoside hydrolases.[6] However, one of the differences of the *A. japonicus* BG from bacterial lichenases was its notable CMCase activity (3.9 U/mg of protein), that was nevertheless much lower than the CMCase activity of true cellulases (*endo*-(1→4)-β-glucanases, EC 3.2.1.4) of *T. reesei* (20–52 U/mg, see Table 2). According to the data acquired in our laboratory during more than 15 years of cellulase research, typical values of CMCase activity for other purified endoglucanases from different fungi fall within the range of 10–60 U/mg. Moreover, the *A. japonicus* BG could not hydrolyze Avicel cellulose at all, while other fungal endoglucanases had notable or at least detectable Avicelase activity, for instance: 0.06–0.20 U/mg in the case of *T. reesei* enzymes (Table 2) or 0.02–0.19 U/mg for endoglucanases from *C. lucknowense*.[8]

Thus, if to consider the substrate specificity, the enzyme isolated from *A. japonicus* seems to be a rather non-typical *endo*-(1→4)-β-glucanase (EC 3.2.1.4) with (i) reduced ability to hydrolyze CMC, (ii) not active against Avicel at all, and (iii) with extremely high specific activity toward β-(1→3)-(1→4)-glucans (barley β-glucan and lichenan), which is comparable in value with the activity of bacterial lichenases.

Study of the mode of action of *A. japonicus* BG on barley β-glucan showed that the enzyme acts on the polymeric substrate by the *endo*-depolymerizing mechanism (Figs. 4A, 5). The end products of β-glucan hydrolysis under the action of BG were similar to those obtained in the *B. subtilis* lichenase-catalyzed reaction, but they were different from the products of β-glucan hydrolysis catalyzed by endoglucanases (EG I, II, and III) from *T. reesei* (Fig. 6). Both the BG and lichenase acted on the polysaccharide in a regular manner forming only tri- and tetrasaccharides as the end products, while the *T. reesei* enzymes formed a wider spectrum of products (glucose, cellobiose, tri- and/or tetrasaccharides). Further studies revealed the difference in the modes of action of *A. japonicus* BG and bacterial lichenases on β-glucans. While bacterial lichenases split the β-(1→4)-glucoside linkage strictly *after* the β-(1→3)-linkage in β-glucan molecules,[6] the fungal BG acted on the β-(1→4)-glucoside linkage *before* the β-(1→3)-linkage (Fig. 10). So, oligosaccharides formed as products of β-glucan hydrolysis with the *A. japonicus* BG contain the β-(1→3)-glucoside linkage at the non-reducing end,

whereas the lichenase-derived oligosaccharides have the β-(1→3)-linkage at the reducing end.

Peptide fingerprinting of the isolated *A. japonicus* BG using MALDI-TOF MS followed by the MASCOT program search in the protein databases (together with data of de novo peptide sequencing by MS/MS) showed that the enzyme has a high sequence identity (not less than 90–95%) to the *endo*-(1→4)-β-glucanase of *A. kawachii* (*A. niger*) from family 12 of glycoside hydrolases (UniProt entry Q12679) as well as lower sequence similarity to the EglA of *A. niger* (O74705). (See Table 1 and Fig. 3.) Up to this date (October 2005), all known *Aspergilli* glycoside hydrolases of family 12 in the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/) have been classified either as *endo*-(1→4)-β-glucanases (EC 3.2.1.4) or as xyloglucan-specific *endo*-β-(1→4)-glucanases (EC 3.2.1.151). Previously,[30] we have isolated a specific xyloglucanase from the same strain of *A. japonicus* that we used in the current study. It has not possessed CMCase activity, but displayed a trace of β-glucanase activity. Subsequent studies (peptide sequencing, unpublished data) showed that this xyloglucanase has a sequence similarity to two known *Aspergilli* xyloglucanases (UniProt entries O94218 and Q6YBY2),[31,32] but it is different from them, thus representing a third family 12 specific xyloglucanase from *Aspergillus* species. The *A. japonicus* BG described in this paper had no activity against xyloglucan.

Most of known family 12 *Aspergilli* glycoside hydrolases listed in the CAZy database (in particular, *A. kawachii* EG, Q12679, showing the highest sequence identity to our *A. japonicus* BG) have been classified as cellulases (*endo*-(1→4)-β-glucanases, EC 3.2.1.4) according to the sequence similarity of the identified genes to the genes encoding other known family 12 cellulases,[27,29,33–35] while the properties of the enzymes and their substrate specificity have not been studied in detail. High β-glucanase activity (that was 20-fold higher than the CMCase activity) has been reported for the EglA from *A. niger*,[36] but the mode of action of β-glucans has not been studied. We isolated the *A. japonicus* enzyme with an even higher ratio of β-glucanase to CMCase activity (~70). So, extremely high β-glucanase activity could be likely observed also for *A. kawachii* EG (Q12679) and, perhaps, for other family 12 enzymes, if they were isolated and studied. If to consider other (non *Aspergilli*) family 12 glycoside hydrolases, one enzyme (from *C. carbonum*,[20] UniProt entry Q9P8N6) has been classified as β-(1→3)-(1→4)-glucanase (EC 3.2.1.73), although in this case the correct classification may be disputed. Previously, we have isolated EG III (Cel12A) from *C. lucknowense*, which also demonstrated extremely high activity against barley β-glucan (125 U/mg protein), while its cellulase (CMCase) activity was moderate (11 U/mg).[8] All these examples show that lichenase-like *endo*-(1→4)-β-glucanases (cellulases with enhanced β-glucanase activity) belonging to the EC 3.2.1.4 group, as well as specific xyloglucanases (EC 3.2.1.151), are often met in family 12 of glycoside hydrolases, and this phenomenon deserves more attention. Especially, this issue may be addressed to specialists working in the field of molecular biology and gene identification who classify the translated nucleotide sequences as enzymes of a concrete class in protein databases (endoglucanases, cellobiohydrolases, xylanases, etc., which is based solely on sequence similarity) without isolating the enzymes and studying their substrate specificity.

4. Experimental

4.1. Enzyme sources

A culture filtrate of *A. japonicus*, obtained from the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, was used for the isolation of β-glucanase (BG). Purified lichenase of *B. subtilis* was from Megazyme (Australia). Commercial *T. reesei* preparation BioAce (from Dyadic International, Inc., USA) was used for the isolation of *endo*-(1→4)-β-glucanases (EG I, II, and III) and β-glucosidase (BGL1).

4.2. Substrates

Barley β-glucan, tamarind seed xyloglucan, linear, and branched arabinans from beet, potato galactan were purchased from Megazyme (Australia); lichenan from *Cetraria islandica*, laminarin from *Laminaria digitata*, CMC (medium viscosity, Cat. No. C4888), birch glucuronoxylan, polygalacturonic acid, and *p*-nitrophenyl derivatives of β-D-Glc*p*, β-cellobiose, β-lactose, β-D-Xyl*p*, α-D-Xyl*p*, α-L-Ara*f*, α-L-Ara*p* were from Sigma Chemical Co. (USA); Avicel PH 105 cellulose was from Serva (Germany); galactomannan was from the Laboratory of Carbohydrates (Bach Institute of Biochemistry, Russian Academy of Sciences); soluble potato starch was provided by NPO of Starch Products (Russia).

4.3. Purification of enzymes

Chromatographic purification procedures were carried out on a Pharmacia FPLC system (Sweden). Source 15Q (3.6 × 5 cm), Source 15 Isopropyl (1.0 × 10 cm), Phenyl-Superose (0.5 × 5 cm), Mono S (0.5 × 5 cm), and Mono P (0.5 × 20 cm) columns were also from Pharmacia. Desalting and buffer equilibration procedures were carried out on Bio-Gel P-4 columns (Bio-Rad Laboratories, USA).

For the isolation of *A. japonicus* BG, the culture filtrate was desalted and equilibrated with 20 mM piperazine·HCl buffer, pH 6.2. Then, a sample containing 400 mg of protein was loaded on a Source 15Q column

equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0–0.4 M NaCl at a flow rate of 10 mL/min (gradient volume 1600 mL). A protein fraction displaying the BG activity was eluted at 0.04 M of NaCl. To this fraction, $(NH_4)_2SO_4$ was added to a final concentration of 1.7 M, and then it was loaded on a Source 15 Isopropyl column equilibrated with 1.7 M $(NH_4)_2SO_4$ in 50 mM NaOAc buffer, pH 5.0. Proteins were eluted with a reversed linear gradient of 1.7–0 M $(NH_4)_2SO_4$ at a flow rate of 2 mL/min (gradient volume 160 mL). A protein peak containing homogeneous BG (28 kDa) was obtained at 1.1 M of $(NH_4)_2SO_4$.

T. reesei EG I (Cel7A), EG II (Cel5A), EG III (Cel12A), BGL1 (Cel3A) were isolated as described elsewhere.[37,38] The purification scheme combined different types of chromatography on Source 15Q, Mono S, Phenyl-Superose columns as well as chromatofocusing on a Mono P column. All the isolated enzymes were homogeneous according to the SDS-PAGE and isoelectrofocusing data. Authenticity of the enzymes was confirmed by peptide fingerprinting experiments using MALDI-TOF MS of the in-gel tryptic digests of the protein bands after the SDS-PAGE, followed by MASCOT search (http://www.matrixscience.com) in the SWISS-PROT database.[26,28]

The enzyme purity was characterized by SDS-PAGE and isoelectrofocusing. SDS-PAGE was carried out in 12% gel using a Mini Protean II equipment (Bio-Rad Laboratories, USA). Isoelectrofocusing was performed on a Model 111 Mini IEF Cell (Bio-Rad Laboratories, USA). Staining of protein bands was carried out with Coomassie Blue R-250 (Ferak, Germany).

The protein content in samples was determined by the Lowry method using bovine serum albumin as a standard or by measuring the absorption at 280 nm.[39]

4.4. MALDI-TOF and tandem TOF/TOF mass spectrometry of peptides

The in-gel tryptic digestion of the protein bands after the SDS-PAGE was carried out essentially as described by Smith.[40] Trypsin (Promega, modified, 5 μg/mL) in 50 mM NH_4HCO_3 was used for a protein digestion. The resulting peptides were extracted from a gel with 20% aq CH_3CN containing 0.1% CF_3CO_2H and subjected to MALDI-TOF MS.[26] Selected peptides from the mass spectrum of the tryptic digest of the *A. japonicus* BG were analyzed by tandem mass spectrometry in order to determine their sequences de novo. An Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik Gmbh, Germany) was used in the MS experiments.

4.5. Enzyme activity assays

Enzyme activities toward polysaccharide substrates were determined using the bicinchoninic acid assay, adapted for 96-well microplates,[41,42] by measuring the amount of reducing sugars released as a result of the enzymatic reaction with polysaccharide (5 mg/mL) after 15 min incubation of the reaction mixture at 50 °C and pH 5.0. An aliquot (40 μL) of the substrate stock solution (10 mg/mL) in 0.1 M NaOAc buffer, pH 5.0, was mixed with 30 μL of the same buffer, the plate was preincubated for 10 min at 50 °C, and the reaction was initiated by the addition of 10 μL of enzyme solution. For a substrate background control, the substrate solution was prepared in the same way, but 10 μL of the acetate buffer was added to the well instead of enzyme. The plate was incubated on a shaker at 50 °C for 15 min. Then, 10 μL of the reaction mixture was transferred into the well of the second plate, 100 μL of the bicinchoninic reagent (A+B)[41,42] and 90 μL of distilled water were added, the plate was sealed with the sealing film for microplates to prevent evaporation and incubated at 80 °C on a water bath for 40 min. The plate was cooled, and the absorbance at 562 nm was measured against a control using an Anthos Labtec HT2 microplate reader (Austria). The calibration was carried out using D-glucose as a standard. Each enzyme activity measurement was carried out at least in duplicate.

Avicelase activity was determined by analyzing reducing sugars released after 60 min of enzyme reaction with 5 mg/mL Avicel cellulose at 40 °C and pH 5.0.[43]

Enzyme activities toward the *p*-nitrophenyl glycosides were assayed at 40 °C and pH 5.0 as described elsewhere[8,43] using the method adapted for 96-well microplates.

All activities were expressed in International Units, that is, one unit of activity corresponds to the quantity of enzyme hydrolyzing 1 μmol of substrate or releasing 1 μmol of reducing sugars (in glucose equivalents) per 1 min.

Studies of the pH effect on the enzyme activity were carried out as described above, except buffer mixtures containing 0.1 M citric acid and 0.2 M Na_2HPO_4[39] were used for maintaining the necessary pH in the reaction system instead of the acetate buffer.

4.6. Determination of kinetic parameters

K_m and V_{max} values toward β-glucan were determined using the bicinchoninic acid assay as described in the preceding section. The substrate concentration was varied in the range of 0.1–16 mg/mL. All assays were carried out in duplicate. The kinetic constants were determined by fitting experimental data to the Michaelis–Menten equation using Microcal Origin 6.0 software.

4.7. Study of β-glucan hydrolysis by GPC

The molecular weight distribution (MWD) of barley β-glucan hydrolysis products was studied by gel-perme-

ation chromatography (GPC) using a TSKgel G3000SWXL column (0.78 × 30 cm, Toso-Haas, Japan). An HPLC Workstation 700 system (Bio-Rad Laboratories, USA) equipped with a refractometric detector was used in these experiments. The elution was carried out with 50 mM NaOAc buffer, pH 5.0. The column was calibrated with dextrans.

For studying the MWD changes of the polymeric substrate under the action of purified enzymes, the β-glucan solution was mixed with the eluent and the enzyme under study to give 4 mL of the substrate solution (5 mg/mL). The enzyme activity in the reaction system was chosen so that it would provide approximately 1% of hydrolysis in every 7–10 min of the reaction at its initial stage. Hydrolysis was carried out at 40 °C on stirring. In the course of the reaction, 300-μL aliquots were taken, incubated in boiling water bath for 5 min to inactivate the enzyme, centrifuged (3 min at 15,000*g*), and then analyzed by GPC and also for reducing sugars released using the bicinchoninic acid assay.[41,42]

4.8. Analysis of the end products of β-glucan hydrolysis

The end products of barley β-glucan and lichenan (5 mg/mL) hydrolysis were analyzed by HPLC on a Workstation 700 system (Bio-Rad Laboratories, USA) equipped with a refractometric detector, using a Diasorb 130 Amino column (4 × 250 mm, 6 μm) from BioChemMack (Russia) and 7:3 CH_3CN–H_2O as the mobile phase. D-Glucose, cellobiose from Sigma (USA), cellotriose, cellotetraose from Seikagaku (USA) and laminaribiose and laminaritriose from Megazyme (Australia) were used as standards in the HPLC analysis.

To remove sequentially (1→4)- or (1→3)-linked β-D-Glc*p* residues from the non-reducing end of oligosaccharides (representing the products of β-glucan hydrolysis), the end aliquots, obtained after the exhaustive hydrolysis of barley β-glucan or lichenan (10 mg/mL) with the *A. japonicus* BG or *B. subtilis* lichenase, were treated with the purified BGL1 of *T. reesei*. The reaction was carried out at 40 °C and pH 5.0 for 96 h using the β-glucosidase activity loading of 0.12 U/mL (activity against 1 mM *p*-nitrophenyl β-D-glucoside is given; it was measured at 40 °C, pH 5.0).

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