Purification, characterization, cDNA cloning, and expression of a xyloglucan endoglucanase from *Geotrichum* sp. M128[1]

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Received 26 November 2003; revised 9 January 2004; accepted 19 January 2004

First published online 28 January 2004

Edited by Judit Ovádi

Abstract A novel xyloglucan-specific endo-β-1,4-glucanase (XEG), xyloglucanase, with a molecular mass of 80 kDa and a p*I* of 4.8, was isolated from the fungus *Geotrichum* sp. M128. It was found to be an endoglucanase active toward xyloglucan and not active toward carboxymethylcellulose, Avicel, or barley 1,3-1,4-β-glucan. Analysis of the precise substrate specificity using various xyloglucan oligosaccharide structures revealed that XEG has at least four subsites (−2 to +2) and specifically recognizes xylose branching at the +1 and +2 sites. The full-length cDNA encoding XEG was cloned and sequenced. It consists of a 2436-bp open reading frame encoding a 776-amino acid protein. From its deduced amino acid sequence, XEG can be classified as a family 74 glycosyl hydrolase. The cDNA encoding XEG was then expressed in *Escherichia coli*, and enzymatically active recombinant XEG was obtained.

Key words: Xyloglucanase; Xyloglucan; Endo-β-1,4-glucanase

1. Introduction

Xyloglucan is a major hemicellulose polysaccharide found in the primary cell wall of plants. It consists of a cellulose-like backbone chain of β-1,4-linked β-D-Glc*p* residues that are frequently substituted at the C6 position with side chains of α-D-Xyl*p*-(1→6), β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6) or α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6) residues. Xyloglucan associates with cellulose microfibrils by hydrogen bonding, forming a cellulose–xyloglucan network [1,2]. Partial disassembly of the cellulose–xyloglucan network is required for cell expansion and development. Xyloglucan metabolism is thought to have an important role in cell differentiation, cell expansion, and the regulation of plant growth and development [3–7]. Recently, it was reported that xyloglucan integration by xyloglucan endotransglycosylase into the cell wall suppresses cell elongation, whereas that of the xyloglucan oligosaccharide XXXG accelerates elongation [8]. It was proposed that xyloglucan metabolism controls plant cell elongation. In the growing plant cell wall, xyloglucan oligosaccharides may provide positive or negative feedback control during cell elongation. Consequently, it is important to determine the oligosaccharide composition of the xyloglucan polymers.

Glycosidases can be useful tools for analyzing the fine structure of complex carbohydrates through their unique specificities for substrates. The identification and characterization of novel glycosidases are important for ensuring progress in glycotechnology. Recently, we identified a unique oligoxyloglucan-specific β-glycosidase, oligoxyloglucan reducing end-specific cellobiohydrolase (OXG-RCBH), from the fungus *Geotrichum* sp. M128 [9]. OXG-RCBH is an exoglucanase that recognizes the structure of the reducing end of oligoxyloglucan and releases two glucosyl residues from the main chain. Upon further analysis, we determined that *Geotrichum* sp. M128 also produced a xyloglucan-specific glycosidase with endoglucanase activity. In this study, we report the purification, characterization, cloning, and expression of a novel xyloglucan-specific endo-β-1,4-glucanase, xyloglucanase (XEG), produced by *Geotrichum* sp. M128.

2. Materials and methods

2.1. Organism

Geotrichum sp. M128 was isolated from soil from Tsukuba, Japan, and entered into the International Patent Organism Depository (IPOD), National Institute of Advanced Industrial Science and Technology (AIST), Japan (number FERM P-16454).

2.2. Purification of XEG from Geotrichum sp. M128

Geotrichum sp. M128 was cultivated in 4-l cultures containing 0.8% xyloglucan from *Tamarindus indica* (Glyloid, Dainippon Pharmaceutical, Osaka, Japan), 0.8% bactopeptone, 0.2% KH_2PO_4, 0.05% $MgSO_4$, and 0.05% yeast extract, pH 6.0, at 30°C for 6 days. Cells were pelleted, and the supernatant was concentrated by ultrafiltration. The concentrated supernatant was diluted with 25 mM imidazole–HCl buffer, pH 7.4, and loaded onto an anion exchange perfusion chromatography column, Poros 50 HQ (Boehringer Mannheim), equilibrated with 25 mM imidazole–HCl buffer, pH 7.4. Elution was performed with a linear gradient of NaCl (0–0.5 M) in 25 mM imidazole–HCl buffer, pH 7.4. Each fraction was assayed for xyloglucan hydrolysis activity by measuring the amount of reducing sugars using the Nelson–Somogyi method [10–12]. The active fractions were pooled, dialyzed against 25 mM imidazole–HCl buffer, pH 7.4, loaded onto an anion exchange perfusion chromatography column, Poros 50

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[1] Nucleotide sequence data reported in this paper are available in the DDBJ/EMBL/GenBank database under accession number AB116528.

Abbreviations: XEG, xyloglucan-specific endo-β-1,4-glucanase; OXG-RCBH, oligoxyloglucan reducing end-specific cellobiohydrolase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; CMC, carboxymethylcellulose; RACE, rapid amplification of cDNA ends; CBM, carbohydrate-binding module

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00068-7

DEAE (Boehringer Mannheim), equilibrated with 25 mM imidazole–HCl buffer, pH 7.4, and then eluted with a linear gradient of NaCl (0–0.5 M). The active fractions were gel-filtrated with a Sephacryl S-300 column (Amersham Biosciences) and applied to another Poros 50 DEAE column. The final active fraction was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a Voyager Biospectrometry Workstation (Perseptive Biosystems). Operation involved an acceleration energy of 20 kV, linear mode, and positive-ion detection. Sinapinic acid was used as a matrix in 50% acetonitrile at a concentration of 10 mg/ml. Bovine serum albumin was used as an external calibration standard.

2.3. Identification of optimum pH, temperature, and stability

Xyloglucan hydrolysis activity was assayed using the Nelson–Somogyi method [10–12]. To determine the optimum temperature, the enzyme (0.05 U/ml) was incubated with tamarind xyloglucan (2.5 mg/ml) in 50 mM sodium acetate (pH 5.5) at various temperatures. Thermostability was analyzed by incubating the enzyme without substrate in the same buffer for 10 min at various temperatures. The remaining activity was then assayed at 45°C. McIlvaine buffer solutions (pH 2.5–9.0), prepared from 0.2 M disodium hydrogen phosphate and 0.1 M citric acid, were used to identify the optimum pH and to determine pH stability. Optimum pH was examined by incubation with tamarind xyloglucan (2.5 mg/ml) at 45°C for 30 min in McIlvaine buffer solutions. The pH stability was assayed by incubating the enzyme, in the absence of substrate, at 25 or 45°C for 30 min in buffer solutions. The buffer solutions were then adjusted to pH 5.5, and the remaining activity was assayed.

2.4. Substrate specificity of XEG

We tested the hydrolyzing activity of purified XEG towards tamarind xyloglucan, pea xyloglucan, barley xyloglucan, carboxymethylcellulose (CMC), Avicel, and barley 1,3-1,4-β-glucan. Each polysaccharide (5 mg) was incubated in 1 ml of 50 mM sodium acetate (pH 5.5) containing 0.5 U of purified XEG at 45°C for 18 h, and the resulting reducing sugars were measured using the Nelson–Somogyi method. The degree of hydrolysis was calculated from the total carbohydrate content estimated using the phenol sulfuric acid method [13] with glucose as the standard.

Next, the substrate specificity was analyzed using various well-defined xyloglucan oligosaccharide structures prepared from tamarind seed [9,14,15]. Each oligosaccharide (0.2 mg) was incubated in 20 μl of 50 mM sodium acetate (pH 5.5) containing 0.005 U of purified XEG at 45°C. After various incubation times (15, 30, 45, and 90 min), the resulting products were quantified by normal-phase high performance liquid chromatography (HPLC) and analyzed by MALDI-TOF mass spectrometry, using previously described methods [9]. These xyloglucan oligosaccharides were named according to Fry et al. [16]; G and X refer to an unbranched β-D-Glc*p* residue and an α-D-Xyl*p*-(1→6)-β-D-Glc*p* segment, respectively. Subsites were labeled according to the subsite nomenclature proposed by Davies et al. [17].

2.5. N-terminal and internal amino acid sequence analysis

The purified protein was separated by SDS–PAGE and transferred from the gel onto a polyvinylidene difluoride membrane filter (Millipore) in transfer buffer (10 mM CAPS, pH 11, 10% methanol). The amino acid sequence was determined by automatic sequential Edman degradation using a Procise 494 HT Protein Sequencing System (Applied Biosystems). To determine internal amino acid sequences, the purified protein was digested with lysyl endopeptidase (Wako Pure Chemical Industries, Osaka, Japan). The resulting peptide fragments were fractionated by reverse-phase HPLC on a TSKgel ODS-80Ts QA column (Tosoh, Tokyo, Japan), and their N-terminal amino acid sequences were analyzed.

2.6. cDNA preparation

cDNA was prepared from *Geotrichum* sp. M128 as described previously [9]. Total RNA was obtained from *Geotrichum* sp. M128 using a FastRNA Kit-RED (BIO 101). mRNA was purified using an oligo(dT) column and a QuickPrep mRNA purification kit (Amersham Biosciences). cDNA was synthesized from the mRNA using an oligo(dT) primer and a TimeSaver cDNA synthesis kit (Amersham Biosciences).

2.7. cDNA cloning

Degenerate primers were designed from internal amino acid sequences (F1 and F2 from peptide 1, and R1 and R2 from peptide 2): F1, 5′-ATHTAYGCNMGIACIGAYATHGG-3′, F2 (nested primer), 5′-GCNMGIACIGAYATHGGIGGIAC-3′, R1, 5′-TGDATNGCNGTICCISWNGCIGD-3′, and R2 (nested primer), 5′-GCNGTICCISWNGCIGCNACDAT-3′. First, the cDNA fragment was amplified by polymerase chain reaction (PCR) using primers F1 and R1. Subsequently, nested PCR was performed with nested primers F2 and R2. The amplified fragment was subcloned into the T-overhang vector, pGEM-T Easy (Promega). Dideoxy double-stranded sequencing of the cDNA insert was performed using an ABI Prism 310 genetic analyzer (Applied Biosystems), according to the manufacturer's instructions, and the expected amplified fragment was confirmed. The complete nucleotide sequences were determined by 5′- and 3′-RACE (rapid amplification of cDNA ends) using a SMART RACE cDNA amplification kit (Clontech). For 5′-RACE, the following gene-specific primers were designed: R3, 5′-CAGTCTGACCACTGCGTAGGCTG-3′, and R4 (nested primer), 5′-CTGCTTGAACGTCTTGCCGCGGTCG-3′. For 3′-RACE, F3, 5′-AGTACCGCTCGCCCGTCCCCATG-3′, and F4 (nested primer), 5′-CACGGCATACGTCGCTTCGCACG-3′, were used as gene-specific primers.

2.8. Expression of XEG in Escherichia coli

The cDNA fragment encoding the mature region of XEG was amplified by PCR with primers that were designed from 5′ and 3′ sequences corresponding to the N-terminal and C-terminal regions of the mature protein: F5, 5′-GGGAATTCCATATGGTCACGGCCGAGCTCAAGCCCGT-3′, and R5, 5′-GGAAGATCTCTACGTGCCGAGGGCCTGGGCACA-3′. *Nde*I and *Bgl*II sites (underlined) were added to the forward and reverse primer, respectively. The amplified DNA was digested with *Nde*I and *Bgl*II, subcloned into the pET-29a(+) expression vector (Novagen), digested with the same restriction enzymes, and transfected into *E. coli* BL21-CodonPlus(DE3) RP (Stratagene). The transfected cells were cultured, and expression was induced with 0.1 mM isopropyl-β-D-thiogalactose (IPTG) for 16 h at 20°C. The soluble, intracellular recombinant protein was extracted using the BugBuster protein extraction reagent (Novagen) and purified on Poros 50 HQ and Poros 50 DEAE columns, as described above.

3. Results and discussion

3.1. Purification of XEG

XEG activity was found in the supernatant of *Geotrichum* sp. M128 cultures, indicating that it was expressed as a secreted protein. XEG was then purified from the supernatant by liquid chromatography (Table 1). Each fraction was assayed for xyloglucan hydrolysis activity. Throughout these steps, enzymatic activity always appeared as a single peak. The final purified fraction had a xyloglucan hydrolysis activity of 68.0 U/mg protein (Table 1) and no detectable OXG-RCBH activity (data not shown). This fraction was subjected to SDS–PAGE, thus revealing a single 80-kDa band (Fig. 1A). The purified XEG was also analyzed by MALDI-TOF mass spectrometry, which revealed an average molecular mass of 80.5 kDa (Fig. 1B). Chromato-focusing showed that its p*I* was 4.8 (data not shown).

3.2. Identification of optimum pH, temperature, and stability

The effect of pH and temperature on purified XEG was analyzed. The optimum pH was 5.5, and the optimum temperature was 55°C. The pH stability was determined at 25°C or 45°C using McIlvaine buffer solutions at various pHs. XEG was stable between pH 5.5 and 8.5 at 25°C, and between pH 5.5 and 6.5 at 45°C. Its thermostability was analyzed at pH 5.5 by incubating the enzyme at various temperatures for 10 min. More than 90% of activity was retained at 50°C.

Table 1
Purification of XEG from *Geotrichum* sp. M128

Step	Activity[a] (U)	Total protein (mg)	Specific activity (U/mg protein)
Culture supernatant	694.8	868.5	0.8
Ultrafiltration	483.6	134.3	3.6
Poros 50 HQ	303.8	14.1	21.6
Poros 50 DEAE (1st)	128.6	2.4	52.8
Sephacryl S300	96.7	1.5	65.3
Poros 50 DEAE (2nd)	78.2	1.2	68.0

[a]One unit of activity is defined as the amount of enzyme that released 1 μmol of glucose equivalents as reducing sugars from the substrate per minute.

3.3. XEG substrate specificity

The enzymatic activity of purified XEG was tested using tamarind xyloglucan, pea xyloglucan, barley xyloglucan, CMC, Avicel, and barley 1,3-1,4-β-glucan. XEG hydrolyzed detectable amounts of xyloglucan, but not other β-glucans (Table 2), indicating that xylose branching is required for XEG activity. The specific activities for tamarind xyloglucan, pea xyloglucan, and barley xyloglucan were 68, 70, and 3 μmol reducing end/min/mg enzyme, respectively. The results also show that barley xyloglucan is a much poorer substrate than tamarind or pea xyloglucan. The barley xyloglucan backbone has fewer substituted xylose residues than the tamarind or pea xyloglucans [1,18]. Since XEG activity requires xylose residues, barley xyloglucan might have fewer cleavage sites than tamarind or pea xyloglucan.

To further analyze its substrate specificity, various oligosaccharide structures were used; the results are shown in Table 3. It cleaved the reducing side of unbranched Glc residues on most substrates, similar to most other endoglucanases. On the non-reducing side, at least two Glc residues were required for activity, indicating that XEG has at least two subsites on the non-reducing side (−2 to −1). Xylose branching at the −2 site had some effect. On the reducing side, two Glc residues were also required, suggesting that XEG has at least four subsites (−2 to +2). Interestingly, XEG had minor activity towards the substrate XXXGX, producing XXX and GX rather than XXXG and X. The cleavage site was the glycosidic bond of the branched Glc residue, rather than the unbranched Glc residue. Therefore, this branching must inhibit its activity, resulting in very low activity. This suggests that the presence of two Glc residues on the reducing side (+1 to +2) is more important for the activity than a lack of branching at the −1 site. If the lack of branching at the −1 site were more important, XXXGX would be hydrolyzed to XXXG and X. However, the recognition of the +1 and +2 sites is more important. In addition, XEG was not active towards XXXGG, indicating that XEG recognizes the Xyl branching at the +2 site. XEG also seemed to recognize the Xyl branching at the +1 site, because it was less active toward GGGX than toward GGXX. In summary, XEG has at least four subsites (−2 to +2) and recognizes the Xyl branching on the reducing side (+1 and +2 site), while branching at the −1 site decreases the activity.

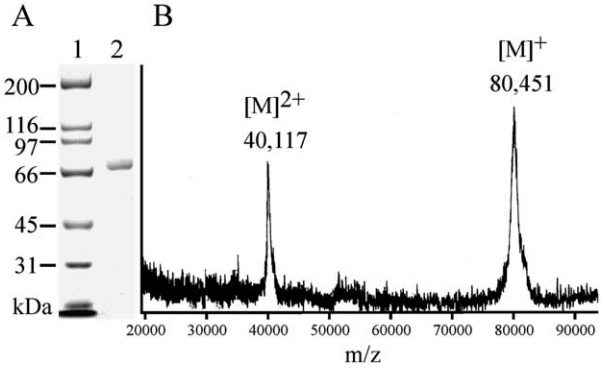

Fig. 1. SDS–PAGE (A) and MALDI-TOF mass spectrometry (B) analysis of purified XEG. A: Lane 1, molecular weight markers; lane 2, final purified fraction. B: The number of charges on the ions is indicated.

3.4. N-terminal and internal amino acid sequences

Purified XEG was analyzed to determine its N-terminal amino acid sequence. The following six amino acids were determined: VTAELK. To analyze internal amino acid sequences, XEG was digested with lysyl endopeptidase, and the resulting peptide fragments were separated by reverse-phase HPLC. The N-terminal amino acid sequences of two peptide fragments were analyzed and determined to be: peptide 1, LIYARTDIGGTY; peptide 2, SIVAASGTAIQS.

3.5. cDNA cloning of XEG

The PCR was carried out using cDNA from *Geotrichum* sp. M128 as a template and degenerate primers (F1 and F2 designed from peptide 1, and R1 and R2 designed from peptide 2). First, cDNA fragments were amplified by PCR with F1 and R1. Subsequently, a nested PCR was performed using primers F2 and R2. The resulting DNA fragment of approximately 600 bp was subcloned and sequenced. The complete cDNA nucleotide sequence was determined by 5′- and 3′-RACE, and the full-length cDNA encoding XEG was cloned. The nucleotide sequence was deposited in DDBJ/EMBL/GenBank under accession number AB116528. The cDNA sequence contained a 2328-bp open reading frame encoding a putative 776-amino acid protein. The deduced amino acid sequence matched the partial amino acid sequences obtained

Table 2
Substrate specificity of XEG on β-glucans

Substrate	Degree of hydrolysis (%)
Xyloglucan (tamarind)	19.6
Xyloglucan (pea)	20.8
Xyloglucan (barley)	3.6
CMC	0.0
Avicel	0.0
Barley 1,3-1,4-β-glucan	0.0

Various β-glucans were incubated with purified XEG, and the resulting reducing sugars were measured by the Nelson–Somogyi method. Degrees of hydrolysis were calculated from total sugars, as measured by the phenol sulfuric acid method.

Table 3
Substrate specificity of XEG on xyloglucan oligosaccharides

Substrate	Specific activity (μmol/min/mg enzyme)	Final products
XXXGXXXG	38	XXXG, XXXG
XXGXXXG	16	XXG, XXXG
GXGXXXG	14	GXG, XXXG
XGXXXG	9	XG, XXXG
GGXXXG	4	GG, XXXG
GXXXG	0	
XXXGXXX	38	XXXG, XXX
XXXGXX	38	XXXG, XX
XXXGX	low	XXX, GX
XXXGG	0	
GGXX	3	GG, XX
GGGX	low	GG, GX

Various oligosaccharide structures were incubated with purified XEG. The resulting products were quantified and analyzed using normal-phase HPLC and MALDI-TOF mass spectrometry.
low: lower than 1 μmol/min/mg enzyme.

from the purified protein. The N-terminal amino acid sequence of the mature protein started at the 21st amino acid residue from the first Met, indicating that 20 amino acids (from −20 Met to −1 Ala) were cleaved from the N-terminus of the mature form. These 20 amino acids are probably a signal sequence. N-terminal proteolytic cleavage resulted in a 756-amino acid protein with an average molecular mass of 80 531 Da, as deduced from the amino acid sequence. This molecular mass resembles that of the native enzyme as determined by MALDI-TOF (Fig. 1B). In addition, the treatment of native XEG with a deglycosylating enzyme, peptide:*N*-glycosidase F, did not affect the results of SDS–PAGE and MALDI-TOF (data not shown). Consequently, although XEG has two potential *N*-glycosylation sites (208Asn–210Ser and 684Asn–686Thr), it is likely that native XEG is not *N*-link glycosylated.

Pauly et al. reported a 23.6-kDa xyloglucanase from *Aspergillus aculeatus* [19] belonging to glycoside hydrolase family 12 (GH12) (http://afmb.cnrs-mrs.fr/CAZY/). It retains β-glucanase activity and is specifically active toward xyloglucan, as is XEG, which was examined in this study. However, no sequence similarity was observed. Although its deduced amino acid sequence shares 48% identity with OXG-RCBH and approximately 30–34% identity with several GH74 proteins, indicating that XEG is part of this family, XEG appears to lack a carbohydrate-binding module (CBM). One member of the GH74 family, *Thermobifida fusca* Xeg74, has been reported to be an inverting enzyme [20], suggesting that all GH74 enzymes use an inverting mechanism. Our time-dependent nuclear magnetic resonance studies of XEG indicate that XEG is also an inverting enzyme (data not shown). Several of the enzymes in the GH74 family that have been characterized exhibit β-glucanase activity, although the substrate specificities differ. *Aspergillus niger* EglC is an endoglucanase with strong activity toward xyloglucan and weaker activities toward CMC and β-glucan [21]. It has a family 1 CBM and shows 31% sequence identity to XEG in the catalytic domain. *Thermotoga maritima* Cel74 is also an endoglucanase, but it is

Fig. 2. Sequence alignment of XEG, OXG-RCBH, *A. niger* EglC, *T. fusca* Xeg74, and *T. maritima* Cel74. Asterisks and periods indicate conserved residues and semi-conserved residues, respectively.

most active toward barley 1,3-1,4-β-glucan and, to a lesser extent, CMC, glucomannan, and xyloglucan [22]. It lacks a CBM and shows 30% sequence identity to XEG. *T. fusca* Xeg74 is a β-glucanase with a family 2 CBM that specifically acts on xyloglucan [20], like XEG reported here. However, the sequence identity of its catalytic domain to XEG is not high (32%). By contrast, OXG-RCBH is a xyloglucan-specific exoglucanase. It lacks a CBM and has the highest sequence identity (48%). It recognizes the reducing end of oligoxyloglucan and produces two glucosyl residue segments [9]. OXG-RCBH also has four subsites (−2 to +2) and recognizes branching at the −1 site. Unlike XEG, the +2 site must be unbranched. It is interesting to note how structural differences between XEG and OXG-RCBH affect the substrate specificity. Fig. 2 shows an amino acid sequence alignment of the catalytic domains of these GH74 family enzymes. Three unique regions for XEG and OXG-RCBH and one unique region for *A. niger* EglC and *T. fusca* Xeg74 that are not found in the others are observed. Their roles are unknown, but they do not seem to correlate with substrate specificity. OXG-RCBH has two unique regions (375Gly–382Gly and 766Lys–789Lys), as compared to the other proteins. It is possible that these regions bestow exoglucanase, rather than endoglucanase, activity on OXG-RCBH. Since no three-dimensional structures are available for GH74 family members, structural analyses are required in order to understand their reaction mechanisms.

3.6. *Expression of XEG in* E. coli

To demonstrate that the protein encoded by the cloned cDNA had enzymatic activity, the cDNA was expressed in *E. coli* strain BL21-CodonPlus(DE3) RP. This strain has additional copies of the genes *argU* and *proL*, which encode tRNAs that recognize the arginine codons AGA and AGG and the proline codon CCC, respectively. The XEG cDNA contains many copies of these codons, particularly CCC. We tried expressing XEG in other host strains (BL21(DE3) and BL21(DE3)pLysS), but the protein was expressed at lower levels than in BL21-CodonPlus(DE3) RP (data not shown). When expression was induced with 0.1 mM IPTG at 20°C, recombinant XEG was found in the BugBuster soluble fraction (Fig. 3, lane 6), as was its enzymatic activity (∼5 U/ml culture), indicating that recombinant XEG was expressed in a soluble enzymatically active form. We then purified recombinant XEG by ion exchange chromatography (Fig. 3, lane 8) and tested its substrate specificity and the effects of pH and temperature on recombinant XEG. The results were similar to those of native XEG. Thus, recombinant XEG will be useful for future analyses, such as structural or enzymatic studies.

3.7. *Conclusion*

In this study, we report a xyloglucan-specific glycosidase, xyloglucanase, belonging to the GH74 family, from *Geotrichum* sp. M128. XEG has at least four subsites (−2 to +2) and specifically recognizes the xylose branching pattern of xyloglucan. It recognizes xylose branching at the +1 and +2 sites, and cleaves the β-link of the +1 site on the non-reducing side. As other enzymes of the GH74 family also exhibit xyloglucan hydrolysis activity, they also have substrate-binding subsites that recognize xylose branching. However, subsites and precise substrate specificity determination using various oligosaccharide structures have only been reported for OXG-RCBH and XEG from *Geotrichum* sp. M128. We believe that these data will contribute to the understanding of substrate recognition and the reaction mechanism of this enzyme family. We are currently analyzing the crystal structure of recombinant XEG and recombinant OXG-RCBH [23]. These studies should lead to an understanding of the mechanism that determines xyloglucan oligosaccharide substrate specificity and might be helpful for improving xyloglucan-specific enzymes.

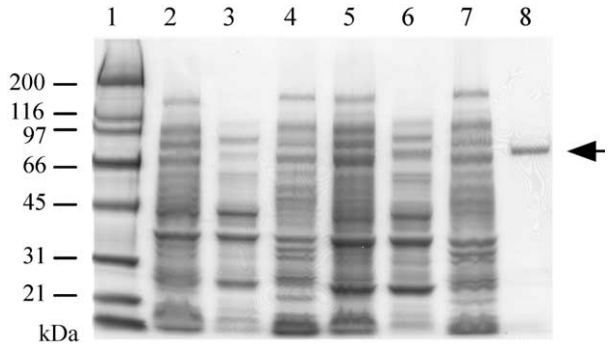

Fig. 3. Recombinant XEG expression in *E. coli*. Recombinant XEG was expressed in *E. coli* strain BL21-CodonPlus(DE3) RP and subjected to SDS–PAGE (7.5–15% gradient gel). The gel was stained with Coomassie brilliant blue. Lane 1, molecular weight markers; lanes 2–4, *E. coli* transformed with pET29a(+) alone (without the XEG cDNA); lanes 5–7, *E. coli* expressing recombinant XEG; lane 8, purified recombinant XEG. Lanes 2 and 5, whole cells; lanes 3 and 6, soluble BugBuster fraction; lanes 4 and 7, BugBuster insoluble fraction. The arrow indicates the running position of recombinant XEG.

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