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ORIGINAL PAPER

Characterisation of a novel *Bacillus* sp. SJ-10 β-1,3–1,4-glucanase isolated from *jeotgal*, a traditional Korean fermented fish

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Abstract A novel β-1,3–1,4-glucanase gene was identified in *Bacillus* sp. SJ-10 (KCCM 90078) isolated from *jeotgal*, a traditional Korean fermented fish. We analysed the β-1,3–1,4-glucanase gene sequence and examined the recombinant enzyme. The open reading frame of the gene encoded 244 amino acids. The sequence was not identical to any β-glucanases deposited in GenBank. The gene was cloned into pET22b(+) and expressed in *Escherichia coli* BL21. Purification of recombinant β-1,3–1,4-glucanase was conducted by affinity chromatography using a Ni-NTA column. Enzyme specificity of β-1,3–1,4-glucanase was confirmed based on substrate specificity. The optimal temperature and pH of the purified enzyme towards barley β-glucan were 50 °C and pH 6, respectively. More than 80 % of activity was retained at temperatures of 30–70 °C and pH values of 4–9, which differed from all other bacterial β-1,3–1,4-glucanases. The degradation products of barley β-glucan by β-1,3–1,4-glucanase were analysed using thin-layer chromatography, and ultimately glucose was produced by treatment with cellobiase.

Keywords β-1,3–1,4-Glucanase · *Bacillus* · β-Glucan

Introduction

β-1,3–1,4-Glucan (β-glucan) is found in the cell wall of Poaceae such as avena, barley, maize, oat, and rice. A total of 70 % of the barley cell wall consists of β-glucan [1]. The plant primary structure is based on the structure and characteristics of hemicelluloses such as mannans, xylans, and β-glucan. Among them, β-1,3–1,4-glucan is the third major hemicellulose [2]. Lichenan (lichenin, moss starch) from lichen (moss) is a representative β-glucan. In addition, β-glucan has been found in algae, bryophytes, fern, and fungi [3–6].

β-1,3–1,4-Glucanase (EC 3.2.1.73) is an endo-β-glucanase that cleaves the β-(1 → 4) in the presence of a β-(1 → 3) linkage in mixed-linkage β-1,3–1,4-glucans. The enzyme produces 3-*O*-cellobiosyl-D-glucose (G3) and 3-*O*-cellotriosyl-D-glucose (G4) from β-glucan. The β-1,3–1,4-glucanase is used commonly in the brewing industry to improve the efficiency of wort filtration and production of beer from barley glucan [7]. Instead of malt enzyme, thermostable β-1,3–1,4-glucanase has been used in the kilning and mashing process due to its heat stability [8]. Also, it is used in the barley-based diets of broiler chicken and piglet to reduce enteritis and increase the digestion–absorption rate [9–11]. Furthermore, β-glucan digested products (oligosaccharides) have been investigated extensively. Jaskari et al. [12] reported that oligosaccharides improve the growth of probiotic bacteria, and Kim et al. [13] found that they have anti-hypercholesterolemic effects. Recently, β-1,3–1,4-glucanase has commonly been used for the production of ethanol from lichenan and barley [14–16]. The addition of the β-1,3–1,4-glucanase reduces the viscosity and increases the mixing of yeast and nutrients.

Bacillus species produce diverse extracellular polysaccharide-degrading and industrially important enzymes [13]. β-1,3–1,4-Glucanase was cloned from *Bacillus* spp., such as *B. halodurans*, *B. subtilis*, *B. brevis*, *B. licheniformis*, and *B. amyloliquefaciens*, and its activity was determined [17–24]. However, most reported enzymes were active at narrow temperature and pH ranges. Therefore, we aimed to identify a β-1,3–1,4-glucanase with high activity under broad conditions. In previous study, we isolated *Bacillus* sp. SJ-10

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producing halotolerant extracellular protease from *joetgal*, a traditional Korea fermented fish, preserved with salt [25]. We considered that this strain produces various tolerant enzymes due to which it can grow at high temperature of 55 °C and high concentrations of 0–14 % NaCl.

In this study, a novel β-1,3–1,4-glucanase gene from *Bacillus* sp. SJ-10 was cloned and expressed, and the biochemical properties of the enzyme were evaluated.

Materials and methods

Bacterial strains, plasmid, media, and growth conditions

Bacillus sp. SJ-10 (KCCM 90078, JCM 15709) was grown in HM (5 % NaCl, 0.5 % yeast extract, 0.5 % proteose peptone, and 0.1 % glucose) medium at 37 °C overnight. *Escherichia coli* DH5α and *E. coli* BL21 were grown in Luria broth (LB) at 37 °C for overexpression. The expression vector used was pET22b(+). The positive transformants were grown in LB supplemented with ampicillin (100 μg/ml) at 37 °C. All strains were mixed with glycerol or DMSO and kept at −70 °C until use.

Sequencing of β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10

Polymerase chain reaction (PCR) was performed using the *Bacillus* sp. SJ-10 chromosomal DNA, universal primers and a PCR kit (EX Taq Kit, Takara, Japan). The universal primers (Bacillus-bg1314-UP, 5′-YCTTATCGTATGAAAC GAGTG-3′; Bacillus-bg1314-RP, 5′-TTTTTTTGTATARC GYACCCA-3′) were derived from GenBank according to conserved *bg1314* gene sequences from *Bacillus* sp. The initial PCR parameters for 30 cycles were: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. PCR was then performed for 35 amplification cycles at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s. Next, the PCR product was reacted with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on an Applied Biosystems model 3730XL (Applied Biosystems, USA). The open reading frame (ORF) was defined using the DNA-Walking Speed-UP Premix Kit (Seegene, Seoul, Korea). The GenBank accession number for the *bg1314* gene sequence of *Bacillus* sp. SJ-10 is JQ782413.

Synthesis of the gene and construction of the expression vector

BSJ-bg1314-UP (5′-GGCCGAATTCGATGTCTTATCGTA TGAAACG-3′) and BSJ-bg1314-RP (5′-GGCCCTCGAG TTTTTTTGTATAGCGCACC-3′) primers were used for PCR to construct the expression vector. PCR was performed for 30 amplification cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The PCR products and pET22b(+) were treated with *Eco*RI and *Xho*I restriction enzymes (Roche, USA). The *bg1314* gene from *Bacillus* sp. SJ-10 and plasmid pET22b(+) were ligated using the T4 ligase kit (Takara, Japan) to construct the recombinant plasmid, pET*bg1314*. pET*bg1314* was transformed into *E. coli* DH5α and finally retransformed into *E. coli* BL21 (*E. coli* pET*bg1314*, KCCM 11264P).

Overexpression and purification of β-1,3–1,4-glucanase

Escherichia coli pETbg1314 was grown until it reached an OD_{600} of 0.4. IPTG (1 mM) was added to induce expression of the recombinant protein at 37 °C for 4 h. Cells were harvested by centrifugation (4,000 rpm, 4 °C, 10 min) and resuspended in 50 mM Tris–HCl (pH 8.0). After sonication, the homogenates were centrifuged (12,000 rpm, 4 °C, 10 min). Pellets were resuspended in 20 mM Tris–HCl (pH 8.0) containing 6 M urea and dialysed into 20 mM Tris–HCl (pH 8.0) for application to the column. Recombinant protein was purified using the Ni-NTA column system. The presence of purified recombinant β-1,3–1,4-glucanase was confirmed by SDS-PAGE. Protein concentrations were determined using the Bradford assay, with bovine serum albumin as the standard.

β-1,3–1,4-Glucanase activity assay

Activity of recombinant β-1,3–1,4-glucanase was assessed using the method of Okeke et al. [27], with some modifications. Briefly, substrates were dissolved in 100 mM sodium phosphate (pH 6). The enzyme and substrate solution were mixed in a 1:1 ratio and reacted at 50 °C for 30 min. DNS (1 ml) was then added to 100 μl of enzyme reaction mixture and boiled for 10 min. The absorbance was measured at 570 nm. One unit (U) of enzyme activity was defined as the amount of protein that produced 1 μmol of D-glucose per minute.

The substrates used were β-D-glucan (from barley, Sigma G6513), 1,3–1,4-β-glucan (from oat), β-1,3-glucan (from *Euglena gracilis*, Sigma 89862), laminarin (Sigma L9634), 1,3–1,6-β-glucan (from yeast), curdlan (Sigma C7821), and cellulose (Sigma C6288). β-D-Glucan was used to evaluate other chemical properties (optimal pH and temperature). To examine the effects of temperature, an enzyme assay was performed after incubating at 10–80 °C (10 °C intervals). Thermostability was evaluated by incubating at 50–80 °C (10 °C intervals) for 60 min (10 min interval sampling), followed by an enzymatic reaction at 50 °C for 30 min. To determine the optimal pH and

stability, the substrates were dissolved in each of the following four buffers: 100 mM potassium chloride–HCl (pH 2), 100 mM sodium acetate (pH 3–6), sodium phosphate (pH 7–8), and glycine–NaOH (pH 9–11).

Determination of enzyme degradation products by thin-layer chromatography

Thin-layer chromatography (TLC), following Apiraksakorn et al. [19], was used to verify the enzymatic reaction products. The enzyme reaction mixture (0.5 µl) was spotted on silica gel 60 TLC plastic sheets (Merck). The developing solvent was composed of *n*-butanol–isopropanol–ethanol–H_2O (2:3:3:2, v/v). The spots were visualised by spraying with 10 % sulphuric acid and then baking at 110 °C for 15–20 min. Standard materials were glucose, cellobiose, cellotriose, and cellotetraose.

Results and discussion

Analysis of the nucleotide and amino acid sequences of *bg1314*

β-1,3–1,4-Glucanase (*bg1314*) from *Bacillus* sp. SJ-10 was composed of 732 nucleic acids and encoded a 244-amino acid protein with a calculated molecular weight of 26.8 kDa. The conserved amino acid motif 'EIDIEF', which is located in the active site of glycosyl hydrolase 16 (GH16) family proteins, contains two glutamic acid residues (E134, E138) required for hydrolytic activity. This motif was present in the *bg1314* gene [7, 28–32]. The *bg1314* nucleotide sequence of *Bacillus* sp. SJ-10 had 95.9 % homology with *B. amyloliquefaciens* FZB42 (CP000560.1), 94.8 % with *B. atrophaeus* 1942 (CP002207.1), 93.3 % with *Bacillus* sp. 289 (FJ031233.1), 93.1 % with *B. subtilis* subsp. *subtilis* 168 (Z46862.1), 92.6 % with *B. subtilis* subsp. *spizizenii* TU-B-10 (CP002905.1), and 90 % with *B. licheniformis* XRK4 (GQ901889.1). The amino acid sequence of β-1,3–1,4-glucanase also has homologies of 96.7, 93.8, 94.3, 91.7, 93.4, and 90.9 % with *B. amyloliquefaciens* FZB42 (ABS75948.1), *B. atrophaeus* 1942 (ADP32279.1), *Bacillus* sp. 289 (ACH85242.1), *B. subtilis* subsp. *subtilis* 168 (CAA86922.1), *B. subtilis* subsp. *spizizenii* TU-B-10 (AEP86905.1), and *B. licheniformis* XRK4 (ACX42225.1), respectively.

Purification of β-1,3–1,4-glucanase

The recombinant plasmid pETbg1314 for overexpression of β-1,3–1,4-glucanase was transformed into *E. coli* BL21(DE3). Protein expression after addition of IPTG was confirmed using SDS-PAGE. β-1,3–1,4-Glucanase (30 kDa), expressed from the pET22b(+) vector, contained an additional His-tag sequence at the C-terminus and was expressed in inclusion bodies. His-tag recombinant β-1,3–1,4-glucanase was effectively purified as a clear single band by Ni-NTA His bind resin (Novagen) (Fig. 1).

Substrate specificity

Various substrates were used to evaluate the substrate specificity of β-1,3–1,4-glucanase. β-1,3–1,4-Glucanase had activity towards β-glucan (60.8 U/mg) from barley and β-glucan (2.8 U/mg) from oat (Table 1). In contrast, β-1,3–1,4-glucanase did not digest β-1,3–1,6-glucan, curdlan, cellulose or dextran. Also, activities against β-1,3-glucan and laminarin were only 1.4 and 0.4 U/mg, respectively. Teng et al. [22] reported that substrate specificity depends on the number of β-1,3 and β-1,4 glycosidic linkages, the branching mode and their distribution in the substrates. β-Glucan from barley and oat is composed of β-1,3 and β-1,4 linkages; and the ratio of β-1,3:β-1,4 linkage is 1:1.9–2.8 in barley and 1:1.5–2.3 in oat [33]. β-1,3-Glucan and curdlan comprised glucose β-1,3 linkages while laminarin and β-glucan from yeast consist of β-1,3 and β-1,6 linkages. Cellulose is a polysaccharide composed of β-1,4 linkages. The four endo-type enzymes that break down β-glucan from barley and oat are β-1,4-D-glucan-4-glucanohydrolase (cellulase, EC 3.2.1.4), β-1,3-D-glucan-3-glucanohydrolase (β-1,3-glucanase, EC 3.2.1.39), β-1,3–1,4-D-4-glucanohydrolase (β-1,3–1,4-glucanase, lichenase, EC 3.2.1.73), and β-1,3(4)-glucanase (laminarinase, EC 3.2.1.6) [17]. Of these, β-1,3–1,4-glucanase can hydrolyse only the β-1,4-glycosidic bond adjacent to β-1,3-glycosidic bond in the mixed glycosidic

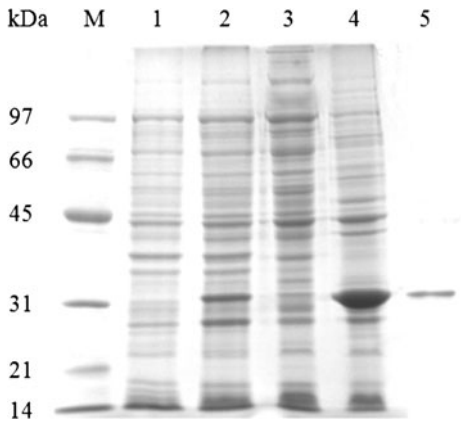

Fig. 1 SDS-PAGE analysis of β-1,3–1,4-glucanase during the purification steps. *Lane M* molecular weight marker, *lane 1* crude extract of *E. coli* pETbg1314 without induction, *lane 2* cell harbouring pETbg1314 after induction with IPTG for 4 h, *lane 3* soluble fraction after induction, *lane 4* inclusion body, *lane 5* purified β-1,3–1,4-glucanase

Table 1 Substrate specificity of the purified β-1,3–1,4-glucanase enzyme and main linkage substrate type

Substrates	Enzyme activity (U/mg)	Main linkage type
β-D-Glucan (barley)	60.8	β-(1,3):(1,4)
1,3–1,4-β-Glucan (oat)	2.8	β-(1,3):(1,4)
β-1,3-Glucan	1.4	β-(1,3)
Laminarin	0.4	β-(1,3):(1,6)
1,3–1,6-β-Glucan (yeast)	–	β-(1,3):(1,6)
Curdlan (*Alcaligenes faecalis*)	–	β-(1,3)
Cellulose	–	β-(1,4)
Dextran	–	α-(1,6):(1,4)

linkages of β-glucan, but cannot hydrolyse the β-1,4-glycosidic bond in cellulose [7]. Therefore, recombinant *Bacillus* sp. SJ-10 is likely a β-1,3–1,4-glucanase. These results also suggest that the activity of β-1,3–1,4-glucanase is proportional to the number of β-1,4-linkage bonds [22]. On the other hand, its hydrolysis activity against barley β-glucan is higher than that against oat β-glucan, despite having the same linkage bond, because of the different proportions of β-glycosidic bonds and the use of low yield β-glucan (8.8 %) from oat as a substrate.

Enzyme assay and stability test

Purified β-1,3–1,4-glucanase (1 μg) was reacted with 0.5 % barley β-glucan under various conditions to determine the optimal temperature and pH. In Fig. 2, the optimal temperature and pH were 50 °C and pH 6, respectively. Moreover, >80 % of enzymatic activity was retained at 30–70 °C and pH 4–9.

The temperature stability test showed that β-1,3–1,4-glucanase was highly stable between 10 and 50 °C, retaining more than 95 % activity. The enzyme activity decreased by 70 and 10 % after a 30-min incubation at 60 and 70 °C, respectively (Fig. 2a). The stability was also evaluated at various pH (3–11) values, and the residual activity was measured under optimal conditions, namely, 50 °C and pH 6. The enzyme was highly stable at pH 3–11 (Fig. 2b).

We next compared the activity of the β-1,3–1,4-glucanase of *Bacillus* sp. SJ-10 to those of other bacteria (Table 2). The optimal conditions for the β-1,3–1,4-glucanase from *Bacillus* spp. were 50–60 °C and pH 6, excluding *B. brevis* and *Bacillus* strain N137.

Recombinant β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10 showed 82 and 84 % activity at pH 4 and 9, while the activity of enzymes from other *Bacillus* spp. was less than 50 % under these conditions. *B. brevis*, *Bacillus* strain N137 and *Rhizopus microspores* enzymes had activities of 100 % (pH 9), 85 % (pH 9) and 98 % (pH 4), respectively. Unlike the β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10, these enzymes showed high activity at narrow pH ranges. Also, *Bacillus* sp. SJ-10 β-1,3–1,4-glucanase activity was 75, 88, 90 and 80 % at 20, 40, 60 and 80 °C, respectively, whereas the enzymes from other *Bacillus* spp. and *Rhizopus* had activities of less than 40 % at 20 and 80 °C (Table 2). Therefore, the β-1,3–1,4-glucanase of *Bacillus* sp. SJ-10 is a novel enzyme with high activity at broad temperature and pH ranges.

To measure the thermostability of β-1,3–1,4-glucanase, the enzyme was incubated at 50–80 °C for 1 h. The residual enzyme activity was more than 95 % at 50 °C for 30 min, and 75, 10 and 10 % at 60, 70 and 80 °C, respectively. Also, the enzyme showed residual activities of 95 and 40 % after incubation at 50 and 60 °C (Fig. 3). The high thermostability of *F. succinogenes* β-1,3–1,4-glucanase was caused by five key amino acids: Gly^{63}, Trp^{54}, Trp^{141}, Trp^{148} and Trp^{203} [34, 35]. *Bacillus* sp. SJ-10 β-1,3–1,4-glucanase also had these five amino acids: Gly^{141}, Trp^{122}, Trp^{180}, Trp^{187} and Trp^{237}. Moreover, Teng et al. [22] reported that five key amino acids, Gly^{113}, Trp^{104}, Trp^{152}, Trp^{159} and Trp^{209}, were found in *B. licheniformis* β-1,3–1,4-glucanase, which had 50 % residual activity after 10 min at 70 °C [22].

Thin-layer chromatography analysis

To examine the products of β-glucan degradation by β-1,3–1,4-glucanase, the hydrolysate was collected after 1-, 6-, 12- and 24-h reactions. The flow rate (Rf) of hydrolysate from β-glucan differed from that of maltotriose and maltotetraose as standard sugar, mainly because G3 and G4 were produced after 1, 6, 12 and 24 h. This demonstrates that β-1,3–1,4-glucanase is an endo-type enzyme (Fig. 4a). Generally, the primary products of β-glucan degradation by β-1,3–1,4-glucanase are triose (G3), tetraose (G4) and a small quantity of cellodextrin-like oligosaccharides (5–10 %) from the polymer regions that contain more than three continuative 4-*O*-linked glucose residues. The rates of G3 and G4 production from barley β-glucan are 52–69 and 25–33 %, respectively [33]. In Fig. 4a, the hydrolysate appeared mainly as two spots (G3, G4). The upper spot likely represents G3 and has a greater intensity than the G4 spot.

Kim et al. found that G3 and G4 have cholesterol-lowering activities. They found that total cholesterol is effectively decreased after injection of G3 and G4 for 6 weeks in diabetic rats [13]. Thus, we predict that G3 and G4 oligosaccharides can be used as bio-health products. The biological effects of hydrolysed oligosaccharides from various polysaccharides have been investigated extensively, but the effects of oligosaccharides from β-glucan

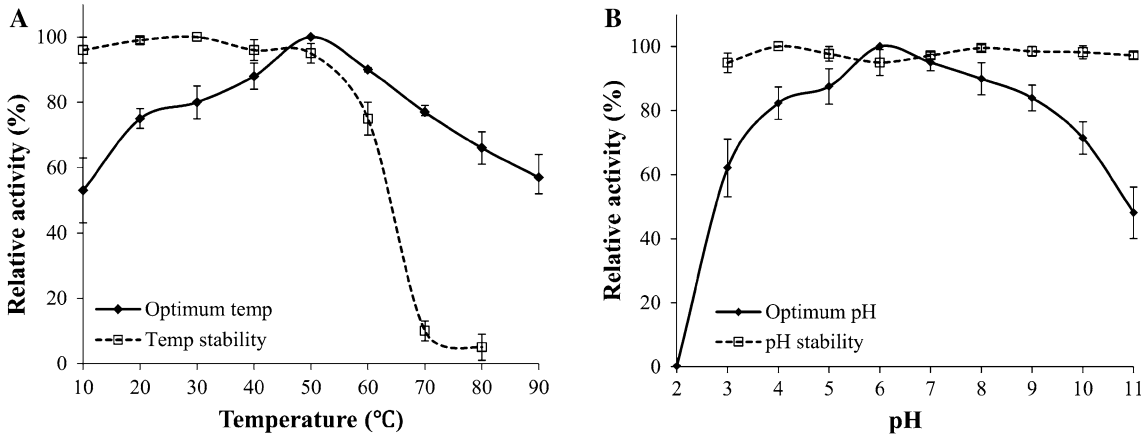

Fig. 2 The effects of temperature (**a**) and pH (**b**) on purified β-1,3–1,4-glucanase. The enzyme was treated at various temperatures and pH values for 30 min. Activity was then assayed at 50 °C for 30 min

Table 2 Comparison of the optimal conditions of the β-1,3–1,4-glucanase with other enzyme from various bacteria under the pH 4, pH 9 and diverse temperature

	Optimal pH	Relative activity (%)		Optimal temp. (°C)	Relative activity (%)					References
		pH 4	pH 9		20 °C	40 °C	50 °C	60 °C	80 °C	
Bacillus sp. SJ-10	6	82	84	50	75	88	100	90	66	This study
B. halodurans C-125	6	18	45	60	–	40	75	100	20	[17]
B. subtilis 168	6	5	10	50	43	80	100	77	–	[18]
B. subtilis GN156	6	5	45	60	18	52	93	100	–	[19]
B. subtilis M139	6.5	0	0	40	35	100	82	54	2	[20]
B. brevis	9	5	100	65	–	40	48	80	32	[21]
B. licheniformis	5.5	15	–	40	–	100	68	48	12	[22]
B. amyloliquefaciens	5–6	50	–	55	–	–	–	–	–	[23]
Alkalophilic *Bacillus* strain N137	7.5	–	85	60–70	5	40	72	100	28	[24]
Rhizopus microsporus var. *microsporus*	5	98	0	50–60	40	80	100	100	–	[26]

– Not reported

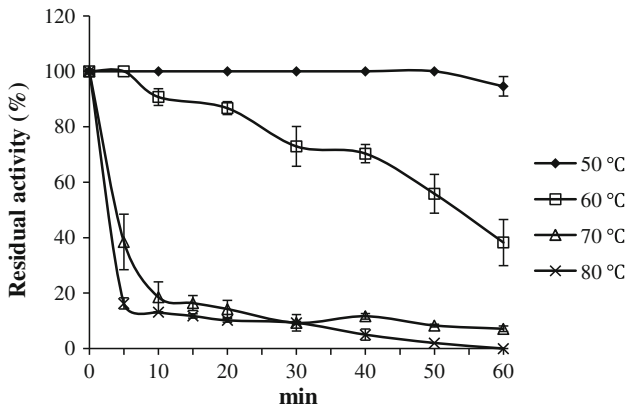

Fig. 3 Thermostability of β-1,3–1,4-glucanase. The enzyme was incubated at 50–80 °C for 1 h. Samples were cooled immediately after different time intervals

remain unclear. Further studies are required to determine the biological effects of β-glucan hydrolysate.

Next, G3 and G4 were incubated with cellobiase from *Aspergillus niger*. Almost all of the oligosaccharides were degraded into glucose (Fig. 4b). Thus, we confirmed that glucose was the ultimate product of β-glucan degradation by β-1,3–1,4-glucanase and cellobiase. Recently, Menon et al. reported the application of lichenase to produce ethanol from lichenan. They found that a high yield of ethanol was produced from complete hydrolysate using the synergistic interaction of lichenase and β-glucosidase. More specifically, lichenase from *Thermomonospora* sp. effectively increased glucose production [14]. Therefore, β-1,3–1,4-glucanase may be applicable to ethanol production in alcohol fermentation using the synergistic interaction with cellobiase.

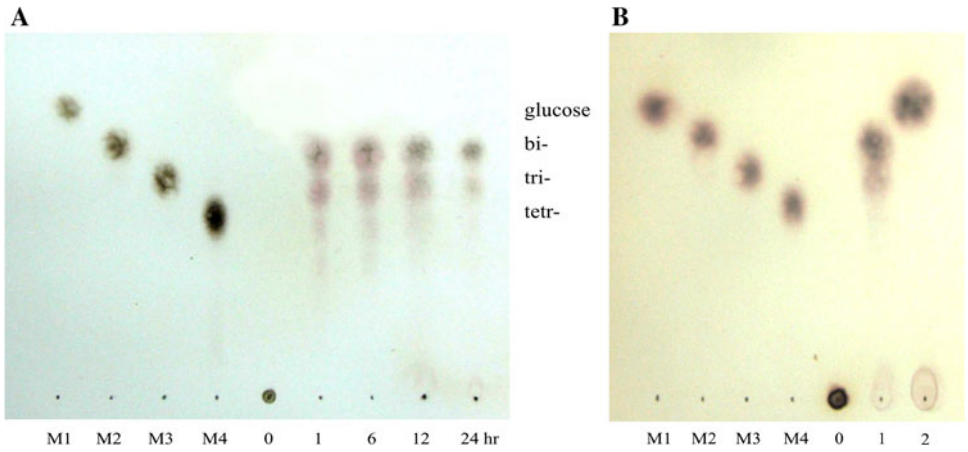

Fig. 4 Thin-layer chromatography of degradation products. **a** Products of barley β-glucan degradation by the β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10 at 0, 1, 6, 12 and 24 h. **b** *Lane 1* product of barley β-glucan degradation by the β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10, *lane 2* product of degradation of the compounds in *lane 1* by the cellobiase from *A. niger*. *M1* glucose, *M2* maltose, *M3* maltotriose, *M4* maltotetraose

In this study, a novel β-1,3–1,4-glucanase with a wide pH and temperature range was identified from *Bacillus* sp. SJ-10, itself isolated from *jeotgal*, and the enzyme was expressed and purified using an *E. coli* overexpression system. Purified β-1,3–1,4-glucanase may be used for production of oligosaccharides and bio-ethanol.

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