Heterologous expression and characterization of two novel glucanases derived from sheep rumen microbiota

De-Ying Gao1,2 · Xiao-Bao Sun1,2 · Ying Fang3 · Bo He1,2,4 · Jun-Hong Wang1,2 · Jian-Xin Liu1,2 · Jia-Kun Wang1,2 · Qian Wang1,2

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Abstract
β-Glucanases are a suite of glycoside hydrolases that depolymerize β-glucan into cellooligosaccharides and/or monosaccharides and have been widely used as feed additives in livestock. In this study, two novel glucanase genes, IDSGluc5-26 and IDSGluc5-37, derived from sheep rumen microbiota, were expressed and functionally characterized. The optimal temperatures/pH of recombinant IDSGLUC5-26 and IDSGLUC5-37 were 50 °C/5.0 and 40 °C/6.0, respectively. Notably, IDSGLUC5-26 showed considerable stability under acidic conditions. Both IDSGLUC5-26 and IDSGLUC5-37 showed the highest activities toward barley β-glucan, with $V_{\text{max}}$ values of 89.96 ± 9.19 µmol/min/mg and 459.50 ± 25.02 µmol/min/mg, respectively. Additionally, these two glucanases demonstrated hydrolysis of Icelandic moss lichenan and konjac gum, IDSGLUC5-26 releasing cellobiose (G2; occupying 17.37% of total reducing sugars), cellotriose (G3; 23.97%), and cellotetraose (G4; 30.93%) from barley β-glucan and Icelandic moss lichenan after 10 min and suggestive of a typical endo-β-1,4-glucanase (EC.3.2.1.4). In contrast, IDSGLUC5-37 was capable of liberating dominant G3 (64.11% or 67.55%) from barley β-glucan or Icelandic moss lichenan, suggesting that the enzyme was likely an endo-β-1,3−1,4-glucanases/lichenase (EC3.2.1.73). These findings describe the expression and characterization of two novel glucanase genes from sheep rumen microbiota. The two recombinant enzymes, particularly the acid-stable IDSGLUC5-26, will be of interest for potential application in food-/feed-additive development.

Keywords Rumen microbe · Glucanase · Heterologous expression · Acid-stable · Substrate hydrolysis

Introduction
As a primary component of hemicellulose, β-glucan is a linear polysaccharide formed by hundreds or even thousands of β-D-glucose residues connected by β-1,3- and β-1,4-glucoside bonds. β-Glucanases are a set of glycoside hydrolases (GHs) that cleave β-glucoside bonds (1–3, 1–4, or 1–6) among main- and/or branched-chain glucans to produce reducing sugars (Li et al. 2020). Endo-glucanase initially acts on internal bonds between sugar rings to liberate cellooligosaccharides, followed by further deconstruction of the intermediates by exo-glucanase and glucosidase to generate cellooligosaccharides with lower degrees of polymerization (DPs) and glucose. According variations in their activities, endo-β-glucanases are further classified into the following four categories: (i) β-1,4-glucanase (EC3.2.1.4), mainly distributed in the GH5 family, which endogenously hydrolyzes β-1,4-d-glycosidic bonds; (ii) β-1,3-glucanase (EC3.2.1.39), mainly distributed in the GH16 family, which endogenously hydrolyzes β-1,3-d-glycosidic bonds; (iii) β-1,3(4)-glucanase (EC 3.2.1.6), mainly distributed in the GH16 family, which endogenously hydrolyzes β-1,3- or β-1,4-d-glycosidic bonds; and (iv) β-1,3,1,4-glucanase (EC 3.2.1.73),
mainly distributed in the GH5 and GH16 families, which specifically cleaves 1,4-β-glycosidic bonds adjacent to 1,3-β-glycosidic bonds (Davies et al. 1995). Additionally, β-glucanase is widely found in the GH6–10/12/24/44/45/6 4/81/128/157/158 families according to the carbohydrate-active enzyme (CAZyme) database (http://www.cazy.org/) (Lombard et al. 2014).

Non-starch polysaccharides (NSPs), including β-glucan, xylan, mannan, pectin, and cellulose, are normally regarded as antinutritional factors in plant-derived feedstuffs. Due to the lack of NSP-degrading enzymes in the digestive tract, single-stomach animals, such as pigs and chickens or even young ruminants with incomplete gastrointestinal function, cannot effectively digest NSP-like feedstuffs. Consequently, increased chyme viscosity directly interferes with the contact between digestive enzymes and their substrates, leading to decreased feed utilization (Beckmann et al. 2006; Romero et al. 2016). Glucanases, particularly endo-β-glucanase, play critical roles in breaking down plant-derived diets. Feed diets supplemented with glucanases reduce the viscosity of digestive-tract contents, improve the morphological structure of the intestine, and promote growth performance of animals. Previous studies reported that glucanase improved the conjugated bile acid fraction in intestinal contents and nutrient digestion in broiler chickens fed a rye-based diet (Mathlouthi et al. 2002; Yu et al. 2002). In recent decades, this enzyme has been used in feed additives and contributed greatly to animal husbandry production.

β-Glucanase is widely found in bacteria, fungi, algae, higher plants, and insects, with a majority of commercial glucanases obtained from microorganisms, especially bacteria and fungi. The main glucanase sources in bacteria include Bacillus spp., such as B. subtilis (Qiao et al. 2009), B. licheniformis (Teng et al. 2007), B. macerans (Heng et al. 1997), B. polymyxa (Guan et al. 2013), and other bacteria, including Fibrobacter succinogenes S85 (Seon et al. 2007), Thermotoga naphthophila RKU-10T (Akram et al. 2020), Citrobacter farmeri A1 (Bai et al. 2016), and Coprococcus eutactus (Alessi et al. 2020). Additionally, Aspergillus niger (Elgharbi et al. 2013), Trichoderma reesei (Geng et al. 2012), and Neocallimastis frontalis (Chen et al. 2014) are common fungi that produce β-glucanases. Furthermore, a large number of rumen microorganisms in the rumen of cattle, sheep, and other ruminants are also believed to produce abundant CAZymes, including β-glucanase (Wang et al. 2013). For example, glucanases from Streptococcus bovis (Ekinci et al. 1997), Clostridium thermocellum (Costa et al. 2014), and Ruminococcus flavofaciens (Mondal et al. 2021) have been partially characterized. However, due to the required long-term adaptation to the complicated and restricted growth environment of the rumen, most rumen microorganisms cannot be purely cultured, making it difficult to directly obtain β-glucanase from rumen using conventional pure-culture methods.

Recently, excavating novel enzymes derived from rumen microbiota from ruminants has become a research hotspot, and newly-developed strategies, such as metagenomics and metatranscriptomics, have been adopted to mine CAZymes (Dai et al. 2015; Shinkai et al. 2016; Comtet-Marre et al. 2017; Jose et al. 2017). Previous studies sequenced and analyzed metagenomic DNA from cow rumen, identifying 27,755 putative carbohydrate-active genes and expressing 90 candidate proteins, of which 57% were enzymatically active against cellulosic substrates (Hess et al. 2011). Additionally, our group employed a metatranscriptomics strategy to discover > 2.38 million unigenes from the rumen microbes of Hu sheep, of which 2.65% were GHs. Among these, functional genes related to cellulose degradation were mainly distributed in the GH3/5/9 families (He et al. 2019a). Subsequently, the enzymatic properties, substrate spectrum, and hydrolytic patterns of two glucanases (Cel5A-h38 and Cel5A-h49) were functionally studied (Cao et al. 2021). Cel5A-h49 is a typical endo-β-1,4-glucanase (EC 3.2.1.4), whereas Cel5A-h38 is a bifunctional enzyme exhibiting both endo-β-1,3 – 1,4-glucanase (EC 3.2.1.73) and exo-cellulbio-hydrolase (EC 3.2.1.91) activities. However, many CAZyme candidates have not been characterized. Therefore, to obtain glucanases with high activities and/or novel catalytic patterns, two glucanase genes (IDSGluc5-26 and IDSGluc5-37) were heterologously expressed in Escherichia coli, and the enzymatic properties and hydrolytic products of the two recombinant enzymes were investigated.

Materials and methods

Materials

The engineered expression bacteria E. coli strains BL21/pET28a/IDSGluc5-26 and BL21/pET28a/IDSGluc5-37 (GenBank No: MT832750/MT832751) were constructed previously by our laboratory (He et al. 2019a). Hydrolysis substrates, including barley β-glucan, Icelandic moss lichenan, p-nitrophenol-β-D-maltopyranoside (pNPC), p-nitrophenyl-β-D-glucopyranoside (pNPG), konjac gum, β-1,3-glucan, and cello-oligosaccharides were purchased from Megazyme (Wicklow, Ireland). Beechwood xylan, xanthan gum, and locust bean gum were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carboxymethylcellulose (CMC-Na), microcrystalline cellulose, isopropyl-thio-β-D-galactopyranoside (ITPG), kanamycin, and culture medium were obtained from Sangon Biotech (Shanghai, China). Phosphoric acid-swollen cellulose (PASC) was prepared from Avicel treated with 85% phosphoric acid according to a previously described method (Zhang et al. 2006).
Signal peptides and catalytic domain of IDSGLUC5-26 or IDSGLUC5-37 were predicted using online software (SignalP5.0; http://www.cbs.dtu.dk/services/signalP) and Pfam (http://pfam.xfam.org/), respectively. The three-dimensional (3D) structure of IDSGLUC5-26 and IDSGLUC5-37 were constructed by homology modeling via SWISS-MODEL (https://swissmodel.expasy.org/) using reported cellulases (PDB: 6GJF and 6XSU) as templates. Amino acid (AA) multiple sequence alignment was using online software (https://www.genome.jp/tools-bin/clustalw and https://esprit.ibcp.fr/ESPript/cgi-bin/ESPr ipt.cgi). To build phylogenetic tree, multiple sequence alignment was conducted by MUSCLE using MEGA 6.0 (Kumar Lab, Temple University, Philadelphia, PA, USA) using default parameters. Phylogeny analysis was performed using the Maximum Likelihood statistical method based on the WAG correction model. The test to assess the phylogeny used was performed by the bootstrap method with 1000 bootstrap replications.

Expression and purification of recombinant enzymes

Recombinant E. coli strains BL21/pET28a/IDSGLuc5-26 and BL21/pET28a/IDSGLuc5-37 were cultured, induced with IPTG, and sonicated as previously described (Sun et al. 2019). Crude enzyme was obtained by centrifugation at 12,000 × g, 4 °C for 10 min, and the supernatant were subjected to affinity purification using a HisTrap FF column (GE Healthcare Biosciences, Pittsburgh, PA, USA). The imidazole gradient in the elution buffer ranged from 20 to 300 mM. Purified IDSGLUC5-26 and IDSGLUC5-37 were analyzed by SDS-PAGE (12% running gel and 4% stacking gel) (Laemmli 1970). After electrophoresis, the gel was stained with Coomassie Brilliant Blue G250 and destained with 15% methanol and 5% acetic acid. The substrate activities of enzymes were analyzed using agar plates containing 0.2% (w/v) barley β-glucan, Iceland moss lichenan, and konjac gum. Proteins (~ 20 U) were dotted on agar plates, incubated at 25 °C for 16 h, followed by staining with 0.1% (w/v) Congo Red for 20 min, solution and destaining with 1 mol/L NaCl until transparent bands appeared.

Circular dichroism

Circular dichroism analysis was conducted using a JASCO CD spectrophotometer under constant N₂ flush (J-1500, Japan) and a 0.1 cm path-length cuvette at wavelengths ranging from 190 to 260 nm. The protein concentration was 0.2 mg/mL in sterile ddH₂O.

Enzyme activity assay

Glucanase activity was determined according to the 3,5-dinitrosalicylic acid (DNS) method (Bailey et al. 1992). Briefly, 15 µL glucanase (~ 12 U) was incubated with 60 µL of 0.5% (w/v) barley β-glucan for 10 min at 50 °C or 40 °C and pH 5.0 or pH 6.0, followed by the addition of 75 µL DNS and boiling for 10 min. After cooling to room temperature, absorbance was determined spectrophotometrically at 540 nm. One unit (U) of glucanase activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to glucose per min. Enzyme concentration was measured by the Bradford method (Bradford 1976) and using bovine serum albumin as the standard. Approximately 12 U of purified enzyme was used for all experiments unless otherwise noted. All assays were performed in quadruplicate.

To determine the optimal pH, purified IDSGLUC5-26 or IDSGLUC5-37 were incubated with 0.5% barley β-glucan in various pH buffers (pH 2.2–8.0, citrate-phosphate buffer; pH 8.0–9.0, Tris-HCl buffer; and pH 9.0–10.0, Na₂CO₃–NaHCO₃ buffer) for 10 min at 50 °C or 40 °C, followed by monitoring of glucanase activity. To evaluate the pH stability of IDSGLUC5-26 or IDSGLUC5-37, the enzymes were pre-incubated in pH buffers (pH 2.2–8.0, citrate-phosphate buffer; pH 8.0–9.0, Tris-HCl buffer; and pH 9.0–10.0, Na₂CO₃–NaHCO₃ buffer) for 1 h at 4 °C, and the residual activities were estimated. The thermal inactivation half-life was determined using the formula t_{1/2} = ln2/kₜ, where kₜ is the first-order kinetic constant (Ó’Fágáin 2003). To determine the optimal temperature for IDSGLUC5-26 or IDSGLUC5-37 activities, the enzymes were incubated with 0.5% barley β-glucan in citrate-phosphate buffer (pH 5.0) for 10 min at between 20 and 80 °C. Thermostability evaluation was carried out by assaying residual activities, after preincubation in citrate-phosphate buffer (pH 5.0) for 1 h at between ~40 and ~60 °C. Aliquots were collected at different time intervals (2, 5, 10, 20, 40, and 60 min) and subjected to estimate glucanase activity. The maximum or initial enzyme activities before preincubation were established as 100%.

Substrate specificity

Kinetic parameters were determined by assaying the activities of purified IDSGLUC5-26 or IDSGLUC5-37 (~ 12 U) against 0.5 mg/mL to 10 mg/mL polysaccharides (barley β-glucan, Iceland moss lichenan, konjac gum, pNPC, CMC-Na, PASC, microcrystalline cellulose, xanthan gum, pNPG, β-1,3-glucan, beechwood xylan, and locust bean gum) under standard conditions (40 °C and pH 6.0) for 15 min. To determine the substrate spectrum for IDSGLUC5-26 or IDSGLUC5-37, the enzymes were incubated with 0.5% substrate in citrate-phosphate buffer (pH 5.0) for 15 min. The heatmap revealed the degradability of...
IDSGLUC5-26 or IDSGLUC5-37 to different substrates. The catalytic activities against barley β-glucan were taken as 100%. The \( K_a \) and \( V_{\text{max}} \) values were estimated by fitting the data to the Michaelis–Menten equation using GraphPad Prism software 8.0 (GraphPad Software, San Diego, CA, USA).

**Substrate hydrolysis**

Purified IDSGLUC5-26 or IDSGLUC5-37 (~ 240 U) was incubated with 0.5% barley barley β-glucan or Icelandic moss lichenan for 48 h at 25 °C, the mixture was withdrawn at 10 min, 30 min, 2 h, 6 h, 12 h, 24 h, and 48 h and heated for 10 min to stop the reaction at 99 °C, centrifuged for 15 min at 25 °C and 10,000 × g, and then subjected to thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analyses. All reactions were performed in triplicate. Glucose (G1), cellobiose (G2), cellotriose (G3), cellooligosaccharides were visualized after spraying with visualization reagent (sulfuric acid/ethanol = 5:95, v/v). Plates were then air-dried in a hood and heated for 10 min at 100 °C to develop the chromatogram.

To further determine the amounts of cellooligosaccharides released by IDSGLUC5-26 and IDSGLUC5-37, HPLC was performed using an LC-1200 device (Agilent Technologies, Santa Clara, CA, USA) equipped with an Asahipak NH2P-50 4E column (Shodex China Co., Ltd., Shanghai, China) and an RID-20 A detector, as described previously (Cao et al. 2021).

**Results**

**Sequence analysis of IDSGLuc5-26 and IDSGLuc5-37**

We obtained two glucanase genes IDSGLuc5-26 (GenBank No: MT832750) and IDSGLuc5-37 (GenBank No: MT832751) from rumen uncultured bacterium from Hu sheep (He et al. 2019a). The open reading frames IDSGLuc5-26 and IDSGLuc5-37 were 915 and 2196 bp, encoding 304 and 731 AAs, respectively. SignalP5.0 prediction revealed that a 25-AA signal peptide was included in IDSGLuc5-26 and IDSGLuc5-37 (Figs. S1 and S2), and a type I dockerin domain was identified at the C-terminus of IDSGLuc5-37.

AA alignment (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the AA sequence of IDSGLuc5-26 shared 95.07% identity with an endo-β-1,4-glucanase from *Ruminococcus albus* (GenBank: WP_155267832.1) and was clustered with another endo-β-1,4-glucanase from *R. albus* (GenBank: WP_002852341.1) (Fig. 1). IDSGLuc5-37 showed only 67.99% identity to an endoglucanase from *Ruminococcus* sp. (GenBank: HAE51778.1) or 65.59% identity to cellulases from unclassified *Ruminococcus* sp. (GenBank: MBR1592273). Two conserved potential catalytic residues, exhibited as GLU135 and GLU222 in IDSGLuc5-26 (Fig. S1) or GLU184 and GLU313 in IDSGLuc5-37 (Fig. S2), were conserved with other glucanases (Santos et al. 2012; Meng et al. 2017). However, neither of them was functionally characterized. Interestingly, IDSGLuc5-37 together with two *Ruminococcus* sp. cellulases were classified into a distinct cluster in GH5, with the closest subgroup being endo-β-1,3–1,4-glucanase (Fig. 1).

**Expression and enzymatic properties of recombinant glucanases**

Two *E. coli* transformants harboring IDSGLuc5-26 and IDSGLuc5-37 were induced at 16 °C with 1 mmol/L IPTG for 16 h. After 6 × His-tagged affinity purification, we observed two distinguished bands of ~33 kDa and ~75 kDa (Fig. 2a, b), which were consistent with their theoretical molecular weights, respectively. Circular dichroism analysis demonstrated that both IDSGLuc5-26 and IDSGLuc5-37 possess α-helix with typical double minima in ellipticity at 208 and 222 nm (Fig. 2c), which was consistent with homology modeling structure (Figs. S1 and S2). Both recombinant IDSGLuc5-26 and IDSGLuc5-37 exhibited hydrolytic activities against barley β-glucan, Icelandic moss lichenan, and konjac gum (Fig. 2d–f) on plates. The optimal reaction temperatures of IDSGLuc5-26 and IDSGLuc5-37 were 50 °C and 40 °C, respectively (Fig. 3a, b), and the optimal pH values were 5.0 and 6.0, respectively (Fig. 3c, d). Both enzymes were relatively stable at < 40 °C and maintained a majority of their activities after pretreatment for 1 h at 40 °C (Fig. 4a, b). However, both enzymes degenerated rapidly at temperatures > 50 °C, with neither IDSGLuc5-26 nor IDSGLuc5-37 active after preincubation for 40 min at 50 °C. IDSGLuc5-26 was stable at pH ranges of ~4.0 to ~8.0 (retained > 70% residual activity) after pretreatment at the indicated pH for 1 h, whereas IDSGLuc5-37 showed stability only between pH 5.0 and pH 6.0 (Fig. 4c, d). The pH-stability assay demonstrated that IDSGLuc5-26 showed a relatively wider pH-resilience range as compared with IDSGLuc5-37. Notably, after preincubation at pH 3.5 or pH 4.0 for 1 h, IDSGLuc5-26 retained 82.49% or 98.41% of its initial activity, respectively, exhibiting a half-life of 3.44 ± 0.14 h at pH 3.5.
Substrate specificities of IDSGLUC5-26 and IDSGLUC5-37

To investigate the substrate spectrum of the two enzymes, we estimated their catalytic activities toward various polysaccharides (Fig. 5). The results showed that both IDSGLUC5-26 and IDSGLUC5-37 were robust in catalyzing barley β-glucan, Icelandic moss lichenan, and konjac gum. Notably, IDSGLUC5-26 also exhibited faint activity against pNPC, CMC-Na, and PASC, whereas IDSGLUC5-37 did not. Both enzymes were inactive on other substrates tested in this study.

We then determined the kinetic parameters of IDSGLUC5-26 and IDSGLUC5-37 toward the three preferable substrates. As shown in Table 1, both glucanases showed the highest activities toward barley β-glucan, with $V_{\text{max}}$ values of $89.96 \pm 9.19 \mu\text{mol/min/mg}$ and $459.50 \pm 25.02 \mu\text{mol/min/mg}$, respectively. Notably, IDSGLUC5-37 had a $K_m$ of $3.30 \pm 0.49 \text{mg/mL}$. For Icelandic moss lichenan and konjac gum substrates, both IDSGLUC5-26 and IDSGLUC5-37 showed degenerated
catalytic effects as compared with those determined in the presence of barley β-glucan.

**Analysis of hydrolytic products by IDSGLUC5-26 and IDSGLUC5-37**

To understand the hydrolytic processes of IDSGLUC5-26 and IDSGLUC5-37, we first performed TLC to evaluated the time-course concentrations of cello-oligosaccharides released from barley β-glucan and Icelandic moss lichenan. IDSGLUC5-26 generally liberated G3 and G4 from barley β-glucan (Fig. 6a) and Icelandic moss lichenan (Fig. 6c), whereas IDSGLUC5-37 mainly produced G3, followed by G4 and G2, from barley β-glucan (Fig. 6b). Interestingly, IDSGLUC5-37 exclusively generated G3 from Icelandic moss lichenan (Fig. 6d). The TLC results suggested two different modes of action modes for the two glucanases.

We then conducted HPLC analysis to investigate the detailed compositions of the hydrolyzed products. The results showed that IDSGLUC5-26 initially hydrolyzed barley β-glucan into G1 through G5 (Fig. 7). After hydrolysis for 10 min, the concentrations of G1, G2, G3, G4, and G5 were 0.56 ± 0.06 mmol/L (occupying 14.48% of total reducing sugars), 0.67 ± 0.09 mmol/L (17.37%), 0.93 ± 0.43 mmol/L (23.97%), 1.20 ± 0.09 mmol/L (30.93%), and 0.51 ± 0.09 mmol/L (13.24%), respectively (Fig. 7a, b). Next, G3 and G4 concentrations increased rapidly, G2 remained unchanged, and G1 and G5 concentrations decreased over time. After a 48-h reaction, G3 and G4 presented as the predominant cello-oligosaccharides, yielding 8.45 ± 0.56 mmol/L (57.56%) and 4.88 ± 0.60 mmol/L (33.21%), respectively, along with trace amounts of G2 (6.97%) and G5 (2.25%). The hydrolytic pattern of Icelandic moss lichenan was similar to that of barley β-glucan (Fig. 7c, d). In contrast, IDSGLUC5-37 mainly released G3 from both barley β-glucan and Icelandic moss lichenan (Fig. 8), with maximum G3 concentrations of 13.51 ± 0.19 mmol/L (64.11%) and 4.82 ± 0.01 mmol/L (67.22%), respectively, yielded after decomposition for 10 min. Interestingly, the G4 concentrations also peaked at 10 min and then remained unchanged, which was similar to G3 throughout the reaction (Fig. 8b, d). Additionally, trend graphs of G1, G2, and G5

![Graphs showing enzyme activity](image_url)
Fig. 4 Thermostability and pH stability of recombinant glucanases. Thermostability, IDSGLUC5-26 (a) and IDSGLUC5-37 (b), pH stability of IDSGLUC5-26 (c) and IDSGLUC5-37 (d). The initial activities before preincubation were taken as 100%. Data represent the mean ± SD (n = 4).

Fig. 5 Heat map of substrate spectrum for IDSGLUC5-26 and IDSGLUC5-37. Each polysaccharide substrate and its proposed degrading-enzyme (with relevant enzyme commission No.) are indicated below or above the heat map. The catalytic activities against barley β-glucan were taken as 100%.
produced by IDSGLUC5-37 were generally consistent with those by IDSGLUC5-26 (Fig. 7).

### Discussion

Mammalian herbivores, such as cattle and sheep, directly digest plant-derived feedstuffs mainly through rumen microorganisms in order to obtain nutrition and energy. The faunal composition of rumen microorganisms includes rumen bacteria, rumen protozoa, and fungi. Each gram of rumen content contains $\sim 10^{11}$ bacteria, $\sim 10^3$ to $\sim 10^6$ ciliates, and $\sim 10^2$ to $\sim 10^4$ anaerobic fungi (Makkar et al. 2005). Among rumen microorganisms of adult ruminants, the bacterial structure mainly comprises Firmicutes, Bacteroides, and Proteus phylums (Wang et al. 2016). However, only a small portion of rumen microorganisms can be successfully isolated and functionally characterized using pure-culture technology. Due to the nutritional components and restricted culture conditions, it is difficult to meet the growth needs of microorganisms in extreme environments, such as those of rumen. Even for cultured rumen bacteria, many genera

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**Table 1** Steady-state kinetic constants of IDSGLUC5-26 and IDSGLUC5-37 for various substrates

| Substrate                  | Enzymes          | $V_{\text{max}}$ (µmol/min/mg) | $K_m$ (mg/ml) | $K_{\text{cat}}$ (1/s) | $K_{\text{cat}}/K_m$ (1/s [mg/ml]) |
|---------------------------|------------------|---------------------------------|---------------|------------------------|------------------------------------|
| Barley β-glucan           | IDSGLUC5-26      | 89.96 ± 9.19                   | 13.79 ± 2.11  | 61.09 ± 8.82           | 4.47 ± 0.327                       |
|                           | IDSGLUC5-37      | 459.50 ± 25.02                 | 3.30 ± 0.489  | 657.75 ± 50.65         | 202.08 ± 26.95                     |
| Icelandic moss lichenan   | IDSGLUC5-26      | 10.19 ± 0.819                  | 4.80 ± 0.876  | 6.90 ± 0.821           | 1.46 ± 0.207                       |
|                           | IDSGLUC5-37      | 100.60 ± 6.14                  | 2.93 ± 0.703  | 169.19 ± 18.79         | 60.09 ± 13.96                      |
| Konjac gum                | IDSGLUC5-26      | 15.79 ± 1.29                   | 2.41 ± 0.470  | 10.72 ± 1.24           | 4.56 ± 0.747                       |
|                           | IDSGLUC5-37      | 130.90 ± 8.38                  | 2.57 ± 0.477  | 187.38 ± 16.96         | 75.12 ± 12.13                      |

Data represent the mean ± SD (n = 4)
**Fig. 7** HPLC analysis of hydrolytic products by IDSGLUC5-26. **a** HPLC profiles of hydrolyzed barley β-glucan; **b** Reducing sugars pattern of hydrolyzed barley β-glucan; **c** HPLC profiles of hydrolyzed Icelandic moss lichenan; **d** Reducing sugars pattern of hydrolyzed Icelandic moss lichenan. Data points from the first 6h of each hydrolytic reaction are shown separately to provide a clearer representation of this phase. Data represent the mean ± SD (n=3). G1 glucose, G2 cellobiose, G3 cellotriose, G4 cellotetraose, G5 cellopentaose.

**Fig. 8** HPLC analysis of hydrolytic products by IDSGLUC5-37. **a** HPLC profiles of hydrolyzed barley β-glucan; **b** Reducing sugars pattern of hydrolyzed barley β-glucan; **c** HPLC profiles of hydrolyzed Icelandic moss lichenan; **d** Reducing sugars pattern of hydrolyzed Icelandic moss lichenan. Data points from the first 2h of each hydrolytic reaction are shown separately to provide a clearer representation of this phase. Data represent the mean ± SD (n=3). G1 glucose, G2 cellobiose, G3 cellotriose, G4 cellotetraose, G5 cellopentaose.
glucanases could only hydrolyze glucan-like substrates released G2 and G3 from substrates. Notably, the three Cel5C (or its variant, Cel5A-38) (Ohara et al. 2000; Cao 2021), as well as the endo-β-1,4-glucanases from Ruminococcus sp. (GenBank: WP_155267832.1 and HAE51778.1, respectively), neither the activity nor their properties have been reported (Parks et al. 2018).

**R. albus** and **R. flavefaciens** are two main species in Ruminococcus genus obtained using pure culture. Four glucanase genes, including cel5A (formerly egl) (Ohmiya et al. 1989), cel5B (formerly egIV) (Karita et al. 1993), cel5C (formerly egV) (Obara et al. 2000), and cel5D (formerly egVII) (Taguchi et al. 2004) were found in genome of R. albus F-40. Amino acid alignment indicated that the IDSGLUC5-26 shared 82.7% similarity with Cel5B. The Cel5B was observed to be active towards CMC, however, its substrate specificities and hydrolytic properties were not well understood (Karita et al. 1993). In this study, determination of the substrate spectrum of the enzymes revealed that both IDSGLUC5-26 and IDSGLUC5-37 show robust hydrolytic activity against barley β-glucan, Iceland moss lichenan, and konjac gum, all of which include either β-1,3- or β-1,4-glycoside bonds; however, both are inactive toward β-1,3-glucan (Fig. 5; Table 1), indicating that the two enzymes are glucanases that target β-1,4-glycoside bonds between sugar rings, which was in agreement with substrate specificities of Cel5A, Cel5B, Cel5C, and Cel5D from R. albus. Interestingly, Mondal and colleagues claimed that the RfGH16_21, derived from R. flavefaciens, exhibits robust catalysis against β-1,4-linked glucan, as well as faint activities toward β-1,4-linked laminarin and birchwood xylan (Mondal et al. 2021).

Subsequently, TLC and HPLC analyses clarified the modes of action, revealing that IDSGLUC5-26 mainly produced G2, G3, and G4 (G3 > G4 > G2) as final products from both barley β-glucan and Iceland moss lichenan (Fig. 6a and b; and 7), which is consistent with the hydrolytic patterns of reported endo-β-1,4-glucanases, such as EGV from Talaromyces emersonii (McCarthy et al. 2003), Umcel9y-1 from the paddy soil microbial metagenome (Zhou et al. 2016), and rGH5CelA from termite gut microbiomes (Guerrero et al. 2020). For glucanases from R. albus, Cel5C (or its variant, Cel5A-38) (Obara et al. 2000; Cao et al. 2021) and Cel5D (Taguchi et al. 2004), dominantly released G2 and G3 from substrates. Notably, the three glucanases could only hydrolyze glucan-like substrates with DP ≥ 3. We speculate that these glucanases, including IDSGLUC5-26, are endo-β-1,4-glucanases (EC3.2.1.4) that randomly cleave β-1,4-linkages between glucose rings. Distinct from IDSGLUC5-26, IDSGLUC5-37 dominantly released G3 as the final product, which occupied ~ 64.11% to ~ 67.55% of the total reducing sugars, from both barley β-glucan and Iceland moss lichenan (Figs. 6c, d and 8). According to manufacturer information (https://www.megazyme.com/) and previous studies (Lazaridou et al. 2007; Tang et al. 2012; Mikkelsen et al. 2017; Mondal et al. 2021), both β-glucan and lichenan represent mixed-linked glucans (MLGs), with the ratios of 1,4- to 1,3-β-D linkages in β-glucan and lichenan ranging from ~ 3:1 to ~ 4:1 and ~ 2:1, respectively. Interestingly, after a 10-min hydrolysis, IDSGLUC5-37 generated 13.51 ± 0.19 mmol/L G3 (64.11%) and 3.44 ± 0.19 mmol/L G4 (16.32%) from barley β-glucan, exhibiting a G3:G4 ratio of 3.93, which was also observed in endo-β-1,3−1,4-glucanases from Rhizomucor miehei (Tang et al. 2012), A. niger (Elgharbi et al. 2013), and Malbranchea cinnamomea (Yang et al. 2014). In contrast, IDSGLUC5-37 almost exclusively produced G3 [4.82 ± 0.01 mmol/L (67.55%)] and negligible G1, G2, G4, or G5 from Icelandic moss lichenan. Given the fundamental structure of MLG-like substrates and hydrolytic patterns, we hypothesize that IDSGLUC5-37 is an endo-β-1,3−1,4-glucanase/lichenase (EC 3.2.1.73) specifically restricted to acting on the β-1,4-linkages adjacent to a β-1,3-glycoside bond within main chains of MLGs. Notably, trace amounts of G5 and G2 were also detected during substrate hydrolysis by either IDSGLUC5-26 or IDSGLUC5-37 (Figs. 7 and 8). Moreover, substrate spectrum analysis indicated that in addition to MLGs, IDSGLUC5-37 exhibited considerable activity on konjac gum (Fig. 5; Table 1), which mainly comprises β-1,4-linkages between glucose/glucose, glucose/mannose, or mannose/mannose, suggesting that IDSGLUC5-37 also demonstrates endo-β-1,4-glucanase activity. The appearance of G5 could be attributed to incomplete enzymatic digestion at the early stage, after which G5 was further converted into cellobiooligosaccharides with lower DP s. However, glucose reached its maximum yield at 10 min or 2 h of glucan decomposition by IDSGLUC5-26 or IDSGLUC5-37 and then decreased or even vanished after 12 or 24 h. Although glucose commonly presents as an end product of GH hydrolysis, it is believed to be further consumed by transglycosylation in order to alleviate its inhibitory effect on enzyme activity. Previous studies have reported this phenomenon in glucanases (Zhou et al. 2016; Xiong et al. 2019; Zhang et al. 2019; Cao et al. 2021).

Rumen pH plays a critical role in maintaining the growth and metabolism of rumen microorganisms (Jiang et al. 2019). Generally, the rumen is a neutral or weakly acidic environment (pH 6.0−7.0). Importantly, a low-pH environment (pH 3.5−4.0) is conducive to the microecological
balance of the digestive tract mainly by promoting the proliferation of Lactobacillus sp. and inhibiting the growth of harmful bacteria, such as E. coli (Gibson et al. 2000). Nevertheless, a majority of glucanases obtained from sheep (Ariaeenejad et al. 2020; Cao et al. 2021), goat (Song et al. 2017), cow (Ko et al. 2013), buffalo (Dadheech et al. 2018), and gayal (Wu et al. 2020) rumen microbiota exhibit poor activity and decay easily in acidic environments. For instance, the Cel5A-38, derived from sheep rumen microbes, was found to retain < 10% residual activity after preincubation at pH 4.0 buffer for 1 h (Cao et al. 2021), while the Cel1, derived from buffalo rumen microbes, maintained < 15% of its initial activity, after preincubation at pH 4.0 buffer for 0.5 h (Dadheech et al. 2018). On the other hand, Nguyen et al. (2012) reportedly obtained the glucanase BT-01 derived from the buffalo rumen metagenomic library, with this enzyme capable of maintaining > 70% activity after pre-treatment at pH 2.6 for 3 h. However, the enzyme showed poor thermostability and almost no activity, even at 40 °C (Nguyen et al. 2012). Previous studies indicated that accumulation of negative charges on structural surface of gluconase, which were crucial for conformational stability in acidic conditions, contributed to the reduced optimal pH value and acid-stability (Jia et al. 2012; Niu et al. 2017; Dadheech et al. 2018). For instance, Li and colleagues succeeded in shifting the optimal pH from 6.0 to 5.5 and improving pH stability of CGX 5-1, a mesophilic β-glucanase from B. terquiliensis, by substitutions of Q1E or I133L (Li et al. 2021). Interestingly, combination of these two mutation sites led to better stability below pH 5.0. Additionally, recent studies reported glucanases, such as CoCel5A from Colletotrichum orbishodiphilum (Zhou et al. 2020) and NMgh45 from saline-alkaline lake soil microbial metagenome (Zhao et al. 2018), that demonstrated stability from pH 3.0 to pH 4.0. However, enzymes derived from exogenous microbes, let alone those from extreme conditions, may cause unexpected risks to immune-response-related processes in hosts. In the present study, we found that IDSGLUC5-26 was stable and showed sustained catalytic activity within a pH range of 3.5 to 7.0 (Figs. 3c and 4c). Thus, IDSGLUC5-26 derived from native microbiota in rumen fluid will be of great interest for potential application in feed-additive development. Furthermore, heat-challenge assays demonstrated that both IDSGLUC5-26 and IDSGLUC5-37 are mesophilic enzymes that showed rapid decreases in activities at temperatures > 50 °C (Figs. 3 and 4). Because rumen temperature normally ranges from 38 to 41 °C, the reaction temperatures of IDSGLUC5-26 and IDSGLUC5-37 likely represent microbial accommodation to the gastrointestinal environment of ruminants (Ko et al. 2013; Wang et al. 2015; Dadheech et al. 2018; He et al. 2019a; Wu et al. 2020; Cao et al. 2021). Surprisingly, a highly-thermostable endo-β-1,4-glucanase (PersiCel4) was obtained from a sheep rumen metagenome (Ariaeenejad et al. 2020) and showed both stability and activity at an optimal temperature of 85 °C (retaining > 75% activity) and even after storage for 150 h at 85 °C. Due to its poor thermostability and relatively low activity, the acid-stable IDSGLUC5-26 requires molecular modification to meet the demands of industrial application. Protein-engineering strategies, such as site-directed mutagenesis (He et al. 2019b), directed evolution (Nakatani et al. 2018), and/or molecular cyclization (Sun et al. 2019), can be adopted to improve IDSGLUC5-26 catalytic activity and stability.

In summary, these findings demonstrated the successful mining of novel CAZymes from uncultured rumen microbes and provide a deeper understanding of the catalytic modes of endo-β-1,4-glucanases and lichenase. The two functionally characterized glucanases, and particularly the acid-stable IDSGLUC5-26, will be of great interest for potential feed-additive development.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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