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ARTICLE

Extremely Acidic β-1,4-Glucanase, CelA4, from Thermoacidophilic *Alicyclobacillus* sp. A4 with High Protease Resistance and Potential as a Pig Feed Additive

Yingguo Bai,[†] Jianshe Wang,[†] Zhifang Zhang,[‡] Pengjun Shi,[†] Huiying Luo,[†] Huoqing Huang,[†] Yukun Feng,[†] and Bin Yao*,[†]

[†]Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute and [‡]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China

An acidic endo-β-1,4-glucanase, denoted CelA4 (~48 kDa), was purified from thermoacidophilic *Alicyclobacillus* sp. A4. Two internal peptides of CelA4 showed strong sequence identity to the *Alicyclobacillus acidocaldarius* cellulase precursor and contained the conserved domain and catalytic region of glycoside hydrolase family 51 β-1,4-glucanases, and the N-terminal and three other internal peptides had no close glucanase or cellulase relatives, suggesting that the enzyme might be novel. CelA4 had broad substrate specificity, exhibited maximum activity at 65 °C and pH 2.6, was stable over pH 1.8−7.6, and showed strong resistance to acidic and neutral proteases, notably pepsin. In comparison to the commercial endo-β-1,3−1,4-glucanase, CelA4 was more stable, released more reducing sugar from barley β-glucan, and under simulated gastric conditions, decreased the viscosity of barley−soybean feed to a greater extent. These properties make CelA4 a good candidate as a new commercial glucanase to improve the nutrient bioavailability of pig feed.

KEYWORDS: *Alicyclobacillus* sp. A4; cellulase; β-1,4-glucanase; extremely acidic; high protease resistance

INTRODUCTION

β-Glucans are the major components of cell walls in cereals, such as barley and oat (*1*). In nature, degradation of β-glucans is catalyzed by β-1,4-glucanases, and these are divided into four main categories depending upon the type of glycosidic linkage cleaved: β-1,3−1,4-glucanases (lichenases, EC 3.2.1.73), β-1,4-glucanases (cellulases, EC 3.2.1.4), β-1,3-glucanases (laminarinases, EC 3.2.1.39), and β-1,3(4)-glucanases (EC 3.2.1.6) (*2*).

Microbial glucanases have received a lot of attention because of their extensive application in both industry and agriculture. For example, microbial glucanases have been used to treat pulp waste in the paper industry (*3*), to enhance the extraction of fermentable substances in the beer-brewing and alcohol fermentation industries (*4*), and to increase β-glucan digestibility and improve nutrient bioavailability in the animal feed industry (*5*). Many acidic glucanases from microorganisms have been reported, including an extracellular β-glucanase from *Trichoderma harzianum* (*6*), an extreme acid-tolerant β-glucanase from *Trichoderma koningii* (*7*), an acidic family 7 endo-β-1.3−1,4-glucanase from *Bispora* sp. MEY-1 (*8*), and an endo-β-1.3−1,4-glucanase from *Bacillus subtilis* GN156 (*9*).

*To whom correspondence should be addressed: Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, People's Republic of China. Telephone: +86-10-82106053. Fax: +86-10-82106054. E-mail: yaobin@caas-bio.net.cn.

The genus *Alicyclobacillus* was first established by Wisotzkey et al. (*10*). All *Alicyclobacillus* species are highly thermoacidophilic (defined as optimal growth conditions at 45−60 °C and pH 2.0−5.0) and may be a good source of acidic glucanases. Thus far, three endoglucanases, a family 9 endoglucanase (CelA) (*11*), a thermoacidophilic β-1,4-glucanase (CelB) (*12*) and a cellulase (CelG) (*13*), have been reported from *A. acidocaldarius*, all of which showed acidic pH optima of 4.0−5.5. Because the pH values in the gastrointestinal tract of mammals are varied from 1.5 to 5.5 and most glucanases lost activity at low pH values, an ideal glucanase for potential use in animal feed should have high activity at extreme acidic pH and good stability over a wide pH range. The purpose of our present study was to isolate an extremely acidic β-1,4-glucanase from the thermoacidophilic *Alicyclobacillus* sp. A4.

MATERIALS AND METHODS

Bacterial Strain and Chemicals. *Alicyclobacillus* sp. A4 isolated from a hot spring (*14*) was used in this study and deposited in the China General Microbiological Culture Collection Center (Beijing, China) under registration number CGMCC3147. Trypsin (from bovine, pH 7.6, 14 700 units/mg), α-chymotrypsin (type II from bovine, pH 7.8, ≥40 units/mg), collagenase (type IV from *Clostridium histolyticum*, pH 7.4, 527 units/mg), subtilisin A (type VIII from *Bacillus licheniformis*, pH 7.5, 10 units/mg), β-glucan from barley (G6513), xylan from oat spelt (X0627), mannan (locust bean gum from *Ceratonia siliqua* seeds, G0753), carboxymethylcellulose sodium salt (CMC-Na, C5678), laminarin from *Laminaria digitata* (L9634), soluble starch (S9765), and bovine serum albumin (A8531) were purchased from

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Sigma (St. Louis, MO). Pepsin (1:3000, pH 2.2) was obtained from Amresco (Solon, OH). Commercial endo-β-1,3−1,4-glucanase (r-Bgl) from *Fibrobacter succinogenes* was provided by Xunson Co. (Beijing, China). Yeast extract and peptone were purchased from Oxoid (Basingstoke, U.K.). Unless otherwise stated, all other reagents and chemicals were analytically pure.

Production, Purification, and Sequence Analysis of CelA4. *Alicyclobacillus* sp. A4 CGMCC 3147 was cultivated in medium containing 1 g/L yeast extract, 2 g/L peptone, and 5 g/L barley β-glucan at 60 °C and pH 3.0 for 48 h. The induced culture (5000 mL) was centrifuged at 12000*g* and 4 °C for 10 min to remove cell debris. The culture supernatant was concentrated 25-fold to 200 mL with ultrafiltration membranes with a molecular-weight cutoff size of 6 kDa (Motianmo, Tianjin, China) and 5 kDa (Vivascience, Hannover, Germany), respectively. Ammonium sulfate was added to the concentrated supernatant to 60% saturation, and the solution was centrifuged at 12000*g* and 4 °C for 10 min. The resulting precipitate was resuspended in 10 mL of 20 mM Tris-HCl (pH 8.0, buffer A) and dialyzed against the same buffer for 24 h. The dialyzed solution was then concentrated to 10 mL using PEG8000, then loaded onto a HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia, Uppsala, Sweden), and equilibrated with buffer A with the flow rate of 3.0 mL/min. Elution was achieved using a linear gradient of NaCl from 0 to 1.0 M in buffer A. Fractions with high enzymatic activity (above 70% of the highest activity) using the barley β-glucan as substrate were collected and loaded onto a Superdex 75 GL FPLC column (Amersham Pharmacia). Fractions with the highest enzymatic activity were collected on elution with buffer A at 1 mL/min. The apparent molecular mass of the purified protein, CelA4, was determined using 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), followed by electroblotting on a polyvinylidene fluoride membrane. The protein concentration of the purified enzyme was determined by the Bradford assay, using bovine serum albumin as a standard (*15*).

The amino acid sequence of CelA4 was determined by Edman degradation using an automated protein sequencer (Applied Biosystems, Foster, CA), following trypsin digestion. Sequence alignment with all known proteins in GenBank was conducted using the multiple sequence alignment software ClustalW (*16*).

Enzyme Assay. Glucanase activity of CelA4 was determined by measuring the amount of reducing sugar released from barley β-glucan according to the method by Miller (*17*). Briefly, purified enzyme (1−2 units/mL) and 1% (w/v) barley β-glucan in McIlvaine buffer (0.2 M, pH 2.6) were incubated at 65 °C for 10 min. The reaction was terminated by the addition of 1.5 mL of 3,5-dinitrosalicylic acid (DNS) and determined the absorption value at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose/min from 1.0% (w/v) glucan solution (*18*). Each reaction and its control were run in triplicate.

Substrate Specificity Assay. The substrate specificity of CelA4 for various polysaccharides was determined after incubation at 65 °C for 10 min in McIlvaine buffer (0.2 M, pH 2.6) containing one of the following substrates: barley β-glucan (1%, w/v), CMC−Na (1%, w/v), oat spelt xylan (1%, w/v), mannan (0.5%, w/v), laminarin (1%, w/v), and soluble starch (1%, w/v). Reactions were terminated by adding 1.5 mL of DNS. For a fixed amount of CelA4, the amount of released reducing sugar was estimated as described above.

Effect of the pH and Temperature on Enzyme Activity. The pH optimum for enzymatic activity of purified CelA4 was determined in the pH range of 1.2−7.8 by assessing activity at 65 °C for 10 min. The pH stability of CelA4 was determined by measuring residual enzymatic activity under standard conditions (pH 2.6, 65 °C, and 10 min) after preincubating the enzyme at 37 °C for 1 h at various pH values in the pH range of 1.2−12.0. The buffers used were 0.1 M KCl−HCl for pH 1.2−2.2, 0.1 M McIlvaine buffer for pH 2.6−7.6, 0.1 M Tris−HCl for pH 8.0−9.0, and 0.1 M glycine−NaOH for pH 9.4−12.0. The optimal temperature for CelA4 activity was determined in the temperature range of 35−75 °C by measuring enzyme activity in 0.1 M McIlvaine buffer (pH 2.6) for 10 min. Thermal stability of purified CelA4 was determined by assessing the residual activity under standard conditions (pH 2.6, 65 °C, and 10 min) after incubation of the enzyme at either 60 or 65 °C for different lengths of time in the absence of substrate.

Effect of Proteases on Enzyme Stability. To examine its resistance to different proteases, purified CelA4 (2 μg/mL) was incubated with 200 μg/mL trypsin, α-chymotrypsin, collagenase, or pepsin or 500 μg/mL subtilisin A, at 37 °C and pH optimum corresponding to individual proteases for various durations. Incubation of CelA4 in the absence of protease was the control experiment. Residual activity was determined under standard assay conditions of pH 2.6, 65 °C, and 10 min.

Effect of Various Reagents on Enzyme Activity. To investigate the effect of different metal ions and chemical reagents on the activity of purified CelA4, the enzyme was incubated in McIlvaine buffer (0.1 M, pH 2.6) containing 1 or 10 mM NaCl, KCl, $CaCl_2$, LiCl, $CoCl_2$, $CrCl_3$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $AgNO_3$, $HgCl_2$, ethylenediaminetetraacetic acid (EDTA), SDS, or β-mercaptoethanol at 65 °C. In addition, 3 mM $CuSO_4$ and 30 mM $ZnSO_4$ were also tested. Incubation of CelA4 in the absence of added reagents was the control experiment.

Kinetic Analysis. The K_m and V_{max} values for CelA4 were determined in McIlvaine buffer (0.1 M, pH 2.6) containing 1−10 mg/mL barley β-glucan or CMC−Na, after incubation with purified CelA4 at 65 °C for 5 min. The data were plotted according to the Lineweaver−Burk method (*19*). Each data point represents an average of three independent experiments, and each experiment included three samples.

Enzyme Stability and Hydrolysis of Glucan under Simulated Gastric Conditions. Commercial endo-β-1,3−1,4-glucanase (r-Bgl) as a counterpart showed optimal activity at pH 6.0 and 55 °C and had good thermostability (> 80% activity at 90 °C for 10 min) (*20*, *21*). The stability of CelA4 compared to r-Bgl in simulated gastric fluid (SGF; 0.25 M glycine−HCl containing 2.0 mg/mL NaCl and 3.2 mg/mL pepsin) (*22*, *23*) was determined by incubating the enzymes, separately, in SGF at 37 °C for 20 min at the following pH values: 1.5, 2.0, 2.5, 3.0, 3.5, or 5.5. The pH of the SGF solution was adjusted with 1 M HCl or 2 M NaOH. Each enzyme was used at 1 unit/mL of SGF (as determined at 37 °C and pH 2.6 for CelA4 and at 37 °C and pH 6.0 for r-Bgl). The residual enzyme activity was determined under standard conditions of pH 2.6, 65 °C, and 10 min.

A total of 1 g of barley β-glucan was dissolved in 49 mL of SGF, and the pH was adjusted with 1 M HCl or 2 M NaOH to a final pH of 1.5, 2.0, 2.5, 3.5, 4.0, or 5.5. The solutions were incubated, with agitation, at 37 °C for 30 min, after which the pH values were re-adjusted to the appropriate values. Purified CelA4 (1 mL of a 1 unit/mL solution) or r-Bgl (1 mL of a 1 unit/mL solution) was added to the solutions and incubated, with agitation, at 37 °C for 60 min. Hydrolysis was stopped by the addition of DNS, as described above.

Effect of a pH Gradient on Viscosity under Simulated Gastric Conditions. To determine the effect of a pH gradient on feed viscosity under gastric conditions (*23*), 10 g of barley−soybean feed (87% barley, 10% soybean, and 3% mineral premix) (*24*) was dissolved in 90 mL of SGF and incubated at 37 °C for 60 min. The hydrolysis assay was performed using a pH gradient that simulates gastric conditions after food ingestion as follows: pH 2.0, 20 min; pH 2.3, 20 min; pH 2.8, 20 min; pH 3.8, 10 min; pH 4.6, 10 min; and pH 5.5, 10 min (*25*). pH values were adjusted with 1 M HCl or 2 M NaOH on an ice bath. The effect of Cel4A on the viscosity of barley−soybean feed solution was determined as described by Vlasenko et al. (*26*). The supernatant from the barley−soybean feed/glucanase mixture (10 mL) was filtered using filter paper and then subjected to a viscosity assay at 20 °C. The viscosity of the barley−soybean feed solution in the absence of an enzyme was used as a control. The density was determined as the weight of the 100 mL water or mash. The viscosity of the solution was calculated using the following equation:

$$\mu = (\mu_{water} t \rho)/(t_{water} \rho_{water})$$

where μ is the viscosity, t is the flow time through the viscometer, and ρ is the density.

RESULTS

Purification and Identification of the Glucanase CelA4 from *Alicyclobacillus* sp. A4. Substantial glucanase activity (0.9 unit/mL) was detected in the glucanase-inducing medium of *Alicyclobacillus* sp. A4. After a five-step purification process (summarized in **Table 1**), the final yield of isolated protein was 29%. The purified protein was homogeneous; it exhibited only one band on

Table 1. Summary of the Purification of CelA4 from *Alicyclobacillus* sp. A4

purification steps	volume (mL)	total activity (units)	total protein (mg)	specific activity (units/mg)	recovery (%)
culture supernatant	5000	4460	22.00	202.73	100
hollow fiber membrane	1000	4059	21.34	190.19	81.2
ultrafiltration membrane	200	3693	20.99	175.96	73.9
ammonium sulfate fractionation	6	2769	12.34	224.39	55.4
ion-exchange chromatography	6	2460	0.92	2673.91	49.2
gel filtration chromatography	4	1430	0.34	4205.88	28.6

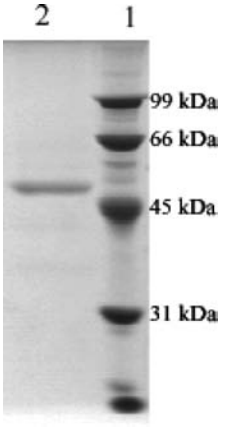

Figure 1. SDS−PAGE analysis of purified protein (CelA4). Lane 1, molecular mass markers; lane 2, purified protein following ultrafiltration, ammonium sulfate fractionation, anion-exchange and gel filtration chromatography.

an SDS−PAGE gel (**Figure 1**). The isolated protein, denoted CelA4, had a molecular mass of approximately 48 kDa (as determined by a comparison to molecular-weight markers on the SDS−PAGE gel) and a specific activity of 4206 units/mg.

The sequence of one of the five internal peptides generated by trypsin digestion (for sequencing of CelA4) was NH_2-A-G-A-Q-P-I-I-T-V-N-A-G-T and shared 83% sequence identity with the amino acid sequence of a cellulase precursor from *A. acidocaldarius* (CAD86595.1) (*12*). Another internal peptide, NH_2-Y-W-E-I-G-N-E-M-Y-G, shared 90% sequence identity with each of the following: a cellulase precursor from *A. acidocaldarius* (CAD86595.1) (*12*), an endoglucanase F precursor from *Fibrobacter succinogenes* (AAC45377.1) (*27*), a cellulase from *F. succinogenes* (BAA10965.1) (*28*), and a cellulase from an environmental DNA sample (CAF22222) (*29*). Both of these internal tryptic peptides shared high identity with glucanases belonging to glycoside hydrolase family 51 (GH51). Multiple sequence alignments using ClustalW indicated that the latter peptide is conserved and functions in the catalytic region (*12*), further indicating that CelA4 should be classified as a member of GH51. The N-terminal amino acid sequence of CelA4, NH_2-D-T-T-T-A-I-A-S-S-T-V-H, and the other three internal peptides, NH_2-I-G-V-D-L-I-A-P-G-T-G-E-D, NH_2-D-P-K-Q-A-Y-T-V-D-L-Q-A, and NH_2-A-G-A-Q-P-I-I-T-V-N-A-G-T, had no close relatives of the glucanase or cellulase, including all those in the glycoside hydrolase (GH) family.

Optimal Conditions for Enzyme Activity. The optimal pH for enzyme activity for purified CelA4 was 2.6 at 65 °C. More than 60% of the maximum activity was retained between pH 1.4 and 5.8 (**Figure 2a**). The enzyme was stable under acid conditions, retaining >80% of its initial activity after incubation at 37 °C in the pH range of 1.8−6.6 for 1 h (**Figure 2b**). The optimal temperature for activity was estimated as 65 °C (**Figure 2c**). Thermal stability of purified CelA4 was also investigated (**Figure 2d**). After incubation at 60 °C for 1 h, the enzyme retained >85% of its initial activity, but all activity was lost after incubation at 65 °C for 1 h.

Substrate Specificity and Kinetic Parameters. Purified CelA4 exhibited the highest enzymatic activity to barley β-glucan (100%), followed by CMC−Na (68.4%), oat spelt xylan (16.4%), and mannan (9.3%). No activity against laminarin and starch was detected.

Kinetic parameters were determined for barley β-glucan and CMC−Na. The K_m and V_{max} values with barley β-glucan as a substrate were 2.00 mg/mL and 5032 μmol min^{-1} mg^{-1}, respectively. With CMC−Na as the substrate, the K_m and V_{max} values were 2.78 mg/mL and 3641 μmol min^{-1} mg^{-1}, respectively.

Effect of Various Reagents and Proteases on Enzyme Activity. The glucanase activity of CelA4 in the presence of different metal ions or chemical reagents is shown in **Table 2**. The enzymatic activity was enhanced by the presence of 1 mM Co^{2+}, Mn^{2+}, or β-mercaptoethanol and was strongly inhibited by SDS. Partial or complete inhibition of enzyme activity was observed in the presence of 10 mM Cr^{3+}, Cu^{2+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^{+}, Ni^{2+} or SDS. At 10 mM, β-mercaptoethanol enhanced the activity by 74.5%. The enzyme retained 91.7 and 86.2% of the initial activity when incubated in the presence of 3 mM Cu^{2+} or 30 mM Zn^{2+}, respectively.

Purified CelA4 was highly resistant to acidic and neutral proteases (**Figure 3**). After incubation with various proteases at 37 °C for 2 h, CelA4 retained >90% of its activity for all proteases tested, except for subtilisin.

CelA4 Stability and Activity in SGF. By comparison to the commercial glucanase (r-Bgl), which is only stable at pH 5.5, CelA4 was highly stable in SGF, retaining >95% activity in the pH range of 2.0−5.5 (**Figure 4a**). CelA4 was also effective at glucan hydrolysis at various pH values, releasing 106, 293, 263, 254, 234, and 219 mg of reducing sugars/g of barley β-glucan at pH 1.5, 2.0, 2.5, 3.5, 4.0 and 5.5, respectively (**Figure 4b**). These values were significantly higher than those for r-Bgl, except for at pH 5.5 ($p < 0.01$).

Effect of a pH Gradient on Viscosity under Simulated Gastric Conditions. When the pH of SGF was adjusted according to the gastric pH gradient after food ingestion using barley−soybean feed, a striking difference ($p < 0.01$) was observed between CelA4 and r-Bgl. CelA4 in fact significantly decreased the feed viscosity by 31.7% ($p < 0.01$), whereas r-Bgl had no effect on feed viscosity.

DISCUSSION

The potential applications for acidic enzymes in several industries have attracted much interest during the past few years. In this study, we isolated an extremely acidic and protease-resistant glucanase, CelA4, from the thermoacidophile *Alicyclobacillus* sp. A4. Among the six peptides of CelA4 sequenced (N-terminal and five internal), two internal tryptic peptides shared high identity with certain GH51 endoglucanases and cellulases and four of them had no close glucanase or cellulase relatives, suggesting that Cel4A might be a new type of enzyme.

To date, three endoglucanases from *A. acidocaldarius* have been reported (*11*−*13*). CelA4 showed some distinct enzymatic properties when compared to these glucanases. For example,

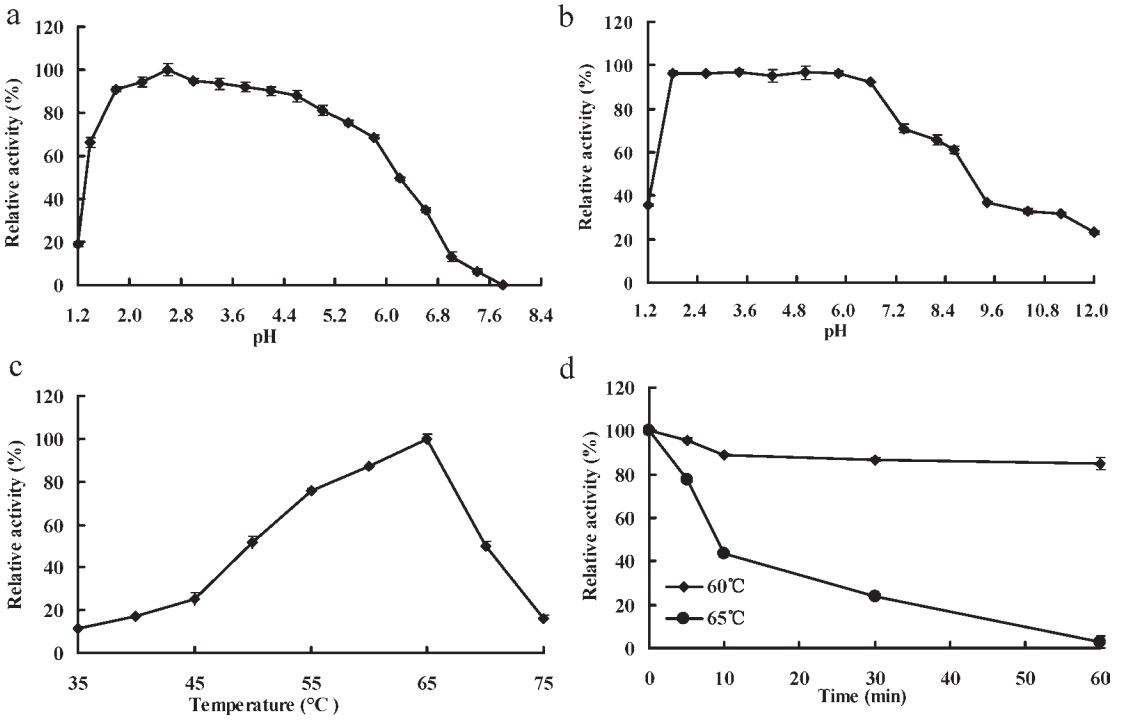

Figure 2. Characterization of purified CelA4. (a) Effect of pH on β-1,4-glucanase activity. Enzyme activity was measured at 65 °C at various pH values in the pH range of 1.2−8.4. (b) Effect of pH on the stability of β-1,4-glucanase activity. After preincubation at 37 °C for 1 h at various pH values, the residual activity of purified CelA4 was measured in McIlvaine buffer (pH 2.6, 65 °C). (c) Effect of the temperature on β-1,4-glucanase activity, measured in McIlvaine buffer (pH 2.6). (d) Thermostability of purified CelA4. The enzyme was preincubated at 60 or 65 °C in McIlvaine buffer (pH 2.6). Aliquots were removed at specific time points, and the residual activity in the same buffer was measured at 65 °C. Each value in the panel represents the mean ± standard deviation (SD) (n = 3).

Table 2. Effect of Metal Ions and Chemical Reagents on the β-1,4-Glucanase Activity of Purified CelA4

	relative activity (%)[a]	
metal ions and reagents	1 mM	10 mM
Na^+	94.2 ± 1.0	99.7 ± 0.9
K^+	92.7 ± 2.1	100.1 ± 0.9
Ca^{2+}	99.5 ± 0.4	90.6 ± 0.7
Li^+	96.1 ± 1.0	90.7 ± 0.3
Co^{2+}	124.1 ± 2.0	90.6 ± 1.8
Cr^{3+}	95.5 ± 1.2	32.1 ± 0.4
Ni^{2+}	105.9 ± 1.9	87.0 ± 0.9
Cu^{2+}	94.9 ± 1.9	56.1 ± 1.1
Mg^{2+}	105.8 ± 0.9	106.5 ± 1.5
Fe^{3+}	91.5 ± 1.1	0
Mn^{2+}	134.9 ± 1.5	ND[b]
Zn^{2+}	99.7 ± 1.3	94.2 ± 0.9
Pb^{2+}	98.7 ± 1.2	15.2 ± 0.4
SDS	13.2 ± 0.3	0
Ag^+	89.0 ± 1.1	38.8 ± 0.7
Hg^{2+}	92.1 ± 1.3	78.7 ± 1.0
EDTA	107.3 ± 2.5	103.8 ± 1.8
β-mercaptoethanol	117.3 ± 1.9	174.5 ± 2.5

[a] Values represent the mean ± SD (n = 3) relative to untreated control samples. [b] ND = not determined.

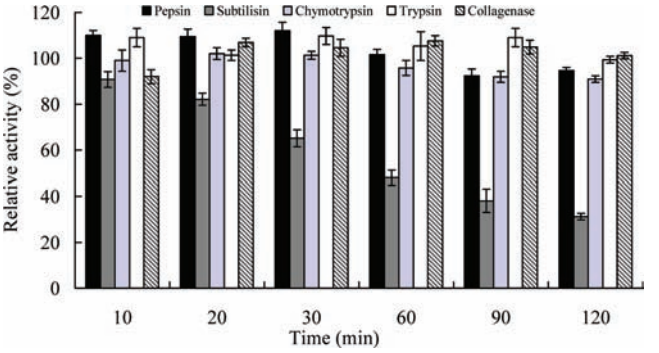

Figure 3. Effect of different proteases on the activity of purified CelA4. The residual activity of CelA4 was measured after incubation with the indicated protease at 37 °C for various lengths of time. Each value in the panel represents the mean ± SD (n = 3).

purified CelA4 showed optimal activity at pH 2.6, exhibited stability over a wide pH range of 1.8−6.6, had broad substrate specificity toward barley β-glucan, cellulose, oat spelt xylan, and mannan, and was highly resistant to pepsin. *A. acidocaldarius* glucanases had pH optima at 4.0−5.5 and was kept stable over a narrower pH range in acidic conditions (*11*−*13*). The β-1,3−1,4-glucanase (Bgl7A) from *Bispora* sp. MEY-1 showed maximal activity at pH 5.0 and was stable over a broader range from acidic to alkaline pH (*8*). However, the specific activities of Bgl7A and CelA4 with CMC−Na as the substrate were 9.8 and 68.4%, respectively, compared to barley β-glucan as the substrate, indicating that CelA4 can more efficiently catalyze the hydrolysis of β-1,4 linkages in CMC−Na. Most β-glucanases used in animal feed are derived from fungi and have acidic pH optima and good stability in acidic conditions. For example, the β-glucanase from *Trichoderma longibrachiatum* ,widely used in animal feed (*30*−*32*), showed high activities in acidic conditions, was kept stable at 70 °C (>65%, 10 min), but lost stability at pH 2.0 or in the presence of pepsin (*30*).

High concentrations of Cu^{2+} and Zn^{2+} have been commonly added to animal feed for several decades because of systemic effects, such as promoting growth, enhancing feed intake, and antimicrobial activity (*33*, *34*). The European Union Council Directive 70/524/EEC limits the total Cu^{2+} content in feed to

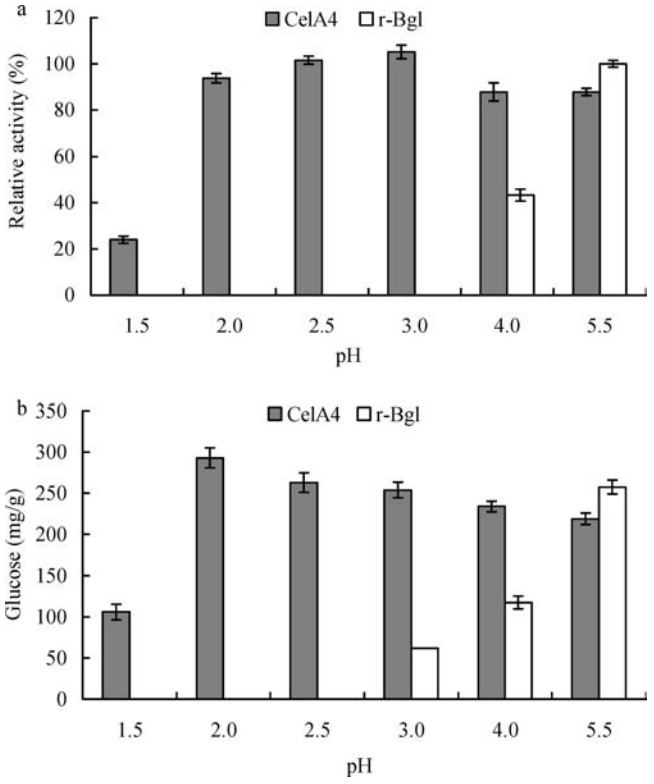

Figure 4. Stability and activity of the glucanases CelA4 and r-Bgl in simulated gastric fluid. (a) Relative activity (mean ± SD, $n = 3$) of CelA4 and r-Bgl at various pH values. (b) Activity of the glucanases (mean ± SD, $n = 3$), measured as milligrams of released glucose per gram of barley β-glucan. Each value in the panel represents the mean ± SD ($n = 3$).

175 mg/kg (*35*). Dybkjaer et al. (*33*) have reported that the addition of 2000 mg of Zn^{2+}/kg of dry matter in piglet feed can efficiently lengthen intake time and promote growth. CelA4 activity was relatively unaffected by the presence of 3 mM Cu^{2+} (approximately 192 mg/kg) or 30 mM Zn^{2+} (approximately 1950 mg/kg), as 92 and 86% of enzymatic activity, respectively, was retained. CelA4, therefore, has potential as an additive for pig feed.

Most protease-resistant glucanases, especially pepsin-resistant glucanases, are obtained from fungi (rather than bacteria) or can be expressed in fungal (but not bacterial) expression systems, such as *Pichia pastoris*. Examples include Bgl7A from *Bispora* sp. MEY-1 (*8*), Bg1 from *Paenibacillus* (*36*), endo-β-1,4-glucanase from *Aspergillus niger* (*37*), and cellulase from *Trichoderma viride* (*38*). The property of protease resistance might therefore be ascribed to a post-translation modification, such as glycosylation. Glycosylation plays an important role in determining enzymatic properties, including thermostability (*39*), isoelectric point, optimum pH (*40*), and resistance to proteases (*41*, *42*). CelA4, isolated from a bacterium, was strongly resistant to various acidic and neutral proteases, including pepsin. This is a good example of β-1,4-glucanase derived from or expressed in a prokaryote that exhibits resistance to pepsin.

β-Glucans are more concentrated in the endosperm and usually comprise between 4 and 8% of total glucans depending upon the type of grain and growing conditions (*43*). β-Glucan in barley cannot be digested by monogastric animals. It causes high viscosity in feed, decreases the digestion rate for other nutritional components, and is therefore a critical anti-nutritional factor (*44*). Moreover, the pig gastrointestinal tract is extremely acidic and contains many proteolytic enzymes. An ideal glucanase should have optimal activity in extremely acidic conditions and be acid-stable and protease-resistant. The pH optimum at extremely acidic pH, high acid stability, and protease resistance of CelA4 would probably allow for the enzyme to function under the conditions present in the digestive tract of animals. Furthermore, the broad substrate specificity of purified CelA4 potentially makes CelA4 more widely applicable than conventional glucanases in the pig feed industry, where the combined activities of different types of glucanase are needed. The high specific activity (4205 units/mg) could lead to cost-effective production. The modest molecular mass of CelA4 (~48 kDa) suggests that overexpression in *P. pastoris* (*45*) is feasible. In summary, CelA4 is a good candidate as a new commercial glucanase to improve nutrient use in the animal feed industry.

ABBREVIATIONS USED

GH, glycoside hydrolase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DNS, 3,5-dinitrosalicylic acid; SGF, simulated gastric fluid.

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