Expression and biochemical characterization of a multifunctional glycosidase from the thermophilic Bacillus licheniformis SR01

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A gene (gkdA) (741 bp) encoding a putative protein of 247 amino acids was cloned from the Bacillus licheniformis SR01. The protein was expressed in Escherichia coli BL21 with a molecular mass estimated by SDS-PAGE of approximately 28.03 kDa and showed a calculating isoelectric point (pI) of 6.42. Structure analysis and function identification showed that the enzyme was a multifunctional glycosidase. Its specific activity was 0.013 U/μg. The recombinant glycosidase showed a maximum activity at 50°C and pH 7.0. It was very stable below 90°C and may have heat activation at higher temperatures. The relative residual activity was still more than 80% after 120 min at pH 5.0–10.0. The enzyme activity was inhibited by Cu2+, Fe2+, Ca2+, Mg2+, Co2+, Li+, SDS and EDTA, activated by Ca2+, and not affected by Mn2+ and K+. Under simulated stomach, and in vitro intestine, conditions, the enzyme retained 80%, and more than 100%, activity, respectively, after incubation for 90 min. The excellent properties of this enzyme, specifically its thermal stability and multifunctional abilities, give it potential application in the field of feed processing and other high-temperature processing industries.

Key Words: characterization; glycosidase; thermophilic Bacillus licheniformis

Introduction

Glycosidases hydrolyse the glycoside bond of various polysaccharide compounds in an endo or exo way. These enzymes exist in almost all organisms and are classified into 125 glycoside hydrolase families based on their nucleotide and amino acid sequences. Family 3 glycosidases are typically able to hydrolyse two or more substrates (Lee et al., 2003). For example, β-D-glucanase, which is isolated from barley, can hydrolyse β-D-glucan and β-D-glucosamine oligosaccharides, as well as the non-reducing end of the released glucose (Harvey et al., 2000).

Multifunctional glycosidases have the ability to simultaneously hydrolyse various substrates. They also show a different catalytic activity under different conditions (Haraldsson et al., 2004). Unlike other enzymes, multifunctional glycosidases do not only maintain catalytic activity but also hydrolysing different substrates, and have a closer integration with the substrate (Fang et al., 2007). Some multifunctional glycosidases have multiple catalytic domains. Palackal et al. isolated a multifunctional glycosyl hydrolase (which possesses mannanase, xylanase and glucanase activities) in an uncultured microbial consortium from ruminant gut. The enzyme consists of two adjacent catalytic domains. One domain belongs to the glycosyl hydrolase family 5, and the other, to family 26 (Palackal et al., 2007). Some multifunctional glycosyl hydrolases have only one catalytic domain but have different catalytic amino acids. Ko et al. isolated and cloned a novel celEdx16 encoding bifunctional endo-/exocellulase...
from Clostridiaceae AN-C16-KBBR, and the active centre analysis showed that Glu174 and Glu298 were the key active amino acid residues for the endocellulase and exocellulase, respectively (Ko et al., 2011). Multifunctional glycosidases have faster and more efficient catalytic properties. A multifunctional glycosidase that hydrolyses lignocellulose, which is developed by the researcher, showed the catalytic activities of xylanase, glucanase and cellulase (Shi et al., 2010). These enzymes can break down oligosaccharides, which are anti-nutritional factors in feed, to improve feed utilisation. Thus, they are often added to feed to break down the oligosaccharides. The enzymes, however, have different properties, making it difficult to achieve co-regulation and limiting feed efficiency improvements. In addition, the feed production process, especially aquatic feed production, often includes a high-temperature process, which slows, or inactivates, the enzymes. Thus, developing a multifunctional and thermally stable glycosidase will be very significant in the feed industry.

Thermophilic bacteria are generally found in hot springs, manure piles, craters, or other extreme environments, and grow at temperatures above 55°C. Because thermophilic bacteria grow in high-temperature environments, their metabolic enzymes can usually tolerate relatively high temperatures. As such, isolating enzymes from thermophilic bacteria is an important means of obtaining thermostable enzymes (Nakazawa et al., 2009). In recent years, researchers have successfully isolated a variety of thermophilic enzymes from thermophilic bacteria with optimum reaction temperatures between 70–90°C, some even up to 100°C or more. For example, endo 1,4-β-xylanase from the thermophilic bacterium Thermotoga has been isolated, and it has an optimum temperature of 105°C (Zverlov et al., 1996). Jiang et al. isolated and cloned the xynB gene, which encodes xylanase, from Thermotoga maritima. The optimum temperature of this xylanase is 90°C, and it was stable up to 100°C (Jiang et al., 1996). Wang et al. cloned α-amylase from Thermococcus siculi HJ2, which had an optimum temperature of 95°C. This enzyme retained 40% of its activity after incubating for 2 h at 100°C (Wang, 2008).

Bacillus licheniformis is a common Bacillus that is usually grown at room temperature. It can be used as a probiotic, in addition to numerous uses in industry and agriculture. The complete genome of Bacillus licheniformis was sequenced in 2008, and the No. 78 gene (YP_006712752.1), which encodes a protein of unknown function, was found (Rey et al., 2004). In this study, we have designed primers based on the sequence of the No. 78 genes from Genbank, cloned the gene (named gkdA) from Bacillus licheniformis SR01 (from Guangxi hot springs, optimal growth temperature 60°C), overexpressed the gene in Escherichia coli BL21, investigated the gene’s function, and characterized the resulting protein.

**Materials and Methods**

**Bacterial strains, plasmids and medium.** The protein Maker, pMD18-T, T4 DNA ligase and restriction endonucleases EcoRI and BamH I from Takara Bio. Raffinose, laminarin and glucanase were purchased from Sigma-Aldrich. All other chemicals were of analytical purity.

Bacillus licheniformis SR01 was kindly supplied by the College of Biological and Chemical Engineering at the Guangxi University of Science and Technology. Vector pMD18-T was used for gene cloning, and plasmid pGEX-4T-3 was used for protein expression. E. coli was used as the host strain for gene cloning and protein expression.

Bacillus licheniformis SR01 was grown on Luria-Bertani (LB) medium at 60°C. The E. coli strains were grown on LB medium or LB agar plates at 37°C with ampicillin (100 µg/ml).

**Gene cloning.** Based on the gene sequence of putative glucanase from Bacillus licheniformis (GenBank: YP_006712752.1), two oppositely oriented PCR primers, LG1s GGAACCCTTGGTGCTGCTATC and LG1a: GAAATTCTACATCATCGCTAATCGC, were designed with restriction enzyme sites (underlined) for BamHI and EcoRI, respectively. Using Bacillus licheniformis SR01 genomic DNA as the template, PCR was performed with a thermocycler at 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min and a final elongation step at 72°C for 10 min. The amplified DNA fragments were digested with BamHI and EcoRI, cloned into their corresponding sites on pGEX-T4-3 and transformed into competent E. coli BL21 cells.

**Sequence analysis and molecular modelling.** Gene and amino acid sequences of the purified enzyme were blasted against the National Center for Biotechnology Information (NCBI) GenBank. Based on amino acid sequence homology, a protein model was built using SWISS-MODEL, and the three-dimensional structure of the protein was modelled based on the reported crystal structure of a “Protein of Unknown Function with Galectin-like Fold” from Bacillus subtilis.

**Protein expression and purification of recombinant enzyme.** E. coli BL21 cells carrying pGEX-4T-gkdA were grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin. Subsequently, the culture was inoculated into 200 ml of fresh LB medium (1:100 dilution) with 100 µg/ml ampicillin and grown at 37°C to an OD600 of 0.5–0.6. Protein expression was induced with 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 h of induction at 30°C, cells were harvested by centrifugation at 12,000 rpm, suspended in 100 mM of phosphate buffer saline (PBS, pH 7.4) and disrupted by sonication on ice with 2-s pulses at 2-s intervals for 30 min. The lysates were centrifuged at 12,000 rpm for 15 min at 4°C to separate the supernatant (soluble protein). The recombinant glutathione-S-transferase was purified according to the manufacturer’s instructions. The eluted fractions were pooled and treated with thrombin protease (40 U/mg) for 16 h at 25°C. After dialysis, the GST protein of the fusion protein was removed. Then, we eluted with a linear NaCl gradient (0–1.0 M) in the same buffer to separate the GST and the thrombin protease from the free gkdA protein, and dialyzed. The purified enzyme samples were analysed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein concentrations
zyme activity was defined as the amount of enzyme released reducing sugar when the enzyme was incubated with various substrates (final concentration, 3 mg/ml) in 100 mM of sodium acetate buffer (pH 3.0–6.0; sodium chloride buffer: pH 7.0–10.0). The pH stability of the enzyme against laminarin was measured by measuring the residual enzyme activity at the optimal temperature and pH for 30 min. The effects of a gastrointestinal environment on the enzyme activity were evaluated by measuring the residual enzyme activity under optimal conditions after pre-incubating the enzyme at 4°C in a simulated in vitro stomach and intestine for 30, 60, 90, and 120 min.

**Results and Discussion**

**Gene cloning and sequence analysis**

The gene encoding a putative protein (gkdA) was successfully cloned into the expression vector pGEX-4T-3 from *Bacillus licheniformis* SR01. The open frame contained 741 bp encoding 247 amino acid residues, and the nucleotide sequence has been submitted to the GenBank database (GenBank: KP869172). The sequence alignment showed that the gene has 99% homology to the template, and only one amino acid location is different from the template. Physical and chemical property analyses showed that the protein has a calculated molecular mass of approximately 28.03 kDa, and a pI of 6.42. Secondary structure analysis of gkdA showed that it contained part of the domain of the glycoside hydrolase family 16, 7, and 32. The 3D model of gkdA was constructed using the SWISS-MODEL server with Protein yesU (PDB ID 1oq1) from *B. subtilis* as the template, with which gkdA shared a 65.44% amino acid sequence identity. The 3D structure is a typical sandwich structure of 12–14 β strands in two sheets with a complex topology, which is similar to the structure of endoglucanase (belonging to the glycoside hydrolase family 16). Other proteins with this structure include xylanase, endoglucanase, glycosyl hydrolase family 16, glycosyl hydrolase family 7, glycosyl hydrolase family 32 and β-d-xylosidase. This result illustrates that the protein may be a multifunctional glycoside hydrolase.

**Protein expression and purification of the recombinant enzyme**

The recombinant plasmid, pGEX-4T-gkdA, was successfully transformed into *Escherichia coli* BL21. The protein was expressed in *Escherichia coli* BL21 with an estimated (by SDS-PAGE) molecular mass of about 28 kDa, which was in agreement with the expected size. A solubilized (by SDS-PAGE) molecular mass of about 28 kDa, which was in agreement with the expected size.
ity analysis showed that the protein was soluble in 0.05 mM IPTG at 30°C, 4 h. The protein was purified to homogeneity by GST affinity chromatography and was characterized with SDS-PAGE (Fig. 1). The protein had a molecular mass of approximately 28 kDa, which was close to the predicted size. The protein concentration was 12.465 μg/ml.

**Substrate specificity and enzyme assay**

The substrate specificity test revealed that the recombinant protein could hydrolyse dextran, xylan, laminarin, pNPG-α-Gal, raffinose, CMCC and filter paper (Table 1). The highest activity was against dextran. The results illustrate that the enzyme was a multifunctional enzyme with xylanase, glucanase, α-galactase and cellulase activity. Although it has a high sequence identity with yesU (putative glucanase) and it belongs to the unknown function protein family 1961, the substrate specificities shows that its function is different than that of glucanase.

A number of multifunction glycosidases displaying glucanase, xylanase and cellulase activity have been reported (Gibbs et al., 2000; Hyeon et al., 2013). These enzymes are mainly used for the hydrolysis of lignocelluloses. A multifunctional hybrid glycosyl hydrolase, discovered in an uncultured microbial consortium from ruminant gut, possessed xylanase, glucanase and mannanase activity (Palackal et al., 2007). A novel multifunctional cellulolytic enzyme (from ruminal bacteria) was also found that breaks down 4-methylumbellifery l-β-α-cellobioside, CMCC, birch wood xylan, oat spelt xylan and 2-hydroxyethyl-cellulose (Ko et al., 2013). However, as far as we know, no multifunctional enzyme possessing glucanase, xylanase, cellulase and α-galactosidase activity has been previously reported, suggesting that this enzyme may be a new multifunctional glycosidase capable of hydrolysing different glycoside bonds. The enzyme could not only hydrolyse lignocellulosic polysaccharides, but also raffinose, stachyose and other α-galactosidase
The multifunctional glycosidase from the thermophilic *Bacillus licheniformis* substrates, giving it a potential application in feed production or other various industries. The activity assay (against laminarin) results showed that its specific activity was 0.013 U/μg.

**Characterization of recombinant enzyme**

The optimal temperature of the enzyme against laminarin was 50°C (Fig. 2a), showing approximately 75% activity at 40°C and 80°C and less than 60% activity at a temperature above 90°C. The optimal temperature of the enzyme was close to that previously reported from *Bacillus* sources, but lower than some thermophilic glucanases from thermophilic and ultra-thermophilic bacteria (Da Silva Aires et al., 2012; Kim et al., 2000; Koutsopoulos et al., 2005; Murray et al., 2001; Ueda et al., 2014). Thermostability assays (Fig. 2b) showed that the enzyme has an excellent stability. The enzyme retained more than 75% activity after incubation for 1 h in a temperature range from 40–70°C, and approximately 60% and 15% activity after 30 min incubation at 90°C and 100°C, respectively. While the glucanase from *Symbiobacterium thermophilum* IAM14863 has the same optimal temperature as this enzyme, it only retained 50% activity after 60 min incubation at 75°C. Even a glucanase (from *Aspergillus niger* US368), with an optimum temperature higher than this enzyme, only retained 50% activity after 30 min incubation at 70°C (Elgharbi et al., 2013). As far as we know, no reported glucanase has retained activity after 30 min incubation at 100°C. Multiple sequence alignments revealed that only one amino acid residue was different from yesU, which may mean that more hydrogen bonds were formed in the protein tertiary structure, resulting in a good heat stability. This result may explain how the microbes could adapt to living in hot springs. Excellent thermal stability of the enzyme is necessary when it is used in feed production, especially aquaculture feeds.

The effect of pH on the enzyme activity (against laminarin) showed that the optimal pH was pH 7.0, which
is close to the pH of an intestinal environment (pH 7.2). It will be more conducive to a catalytic reaction when the enzyme is added to the feed. The enzyme showed approximately 80% activity in a pH range from 5.0 to 9.0, but the activity declined sharply when the pH was lower than 5.0 (Fig. 3a). As for pH stability, the enzyme retained above 80% activity after 1 h and 2 h incubation over a range from pH 5.0 to pH 10.0, but hardly any activity was retained after 1 h incubation at pH 3.0 (Fig. 3b). The results illustrate that the enzyme has a broad pH stability with relatively high activity in an alkaline environment and low activity in a strongly acidic environment. These results agree with those previous reported (Dogra and Sreenivasulu, 2015; Vlasenko et al., 2010; Woo et al., 2014).

The gkdA activity (Fig. 4) was inhibited by Cu²⁺, Fe²⁺, Ca²⁺, Mg²⁺, Mn²⁺, Co²⁺, Li⁺, SDS and EDTA, but most significantly by Cu²⁺ and Fe²⁺, which is similar to the glucanases from B. circulans IAM1165 (Aono et al., 1995), B. clausii NM-1 (Miyaniishi et al., 2003), B. plicatilis (Hara et al., 1997) and sea cucumber Stichopus japonicus (Zhu et al., 2008). SDS is a surfactant and can denature proteins, but the enzyme retained more than 70% activity after it was incubated at 4°C in a PBS buffer (pH 7.4) containing SDS, illustrating that the structure of gkdA was stable. The enzyme activity was markedly stimulated by Ca²⁺, which is similar to endo-β-1,3-glucanase from Trichoderma harzianum (Noronha and Ulhoa, 1996) and Bacillus amyloliquificaciens (Peng and Ou, 2013). Research showed that glucanases have a calcium-binding region far from the active site of the convex surface of the β sheet bend, and that this region plays a role in stabilizing the protein structure (Welfle et al., 1995). The stimulation by Ca²⁺ revealed that gkdA is a metalloenzyme, and Ca²⁺ may be its metal cofactors. Mn²⁺ and K⁺ had no obvious effect on the enzyme. These results mostly agree with previous reports with the exception of glucanase activity inhibited by Mn²⁺ in the studies (Liu et al., 2010; Takehara et al., 1981; Yan, 2008; Zhang et al., 2014). The difference may be due to the structure of gkdA, which has a multifunctional binding region different from the previously mentioned glucanase.

The examination of the gastrointestinal environment on gkdA activity (Fig. 5) showed that gkdA had a high tolerance to gastrointestinal fluids. The enzyme retained approximately 80% and 70% activity after 90 min and 120 min incubation in a simulated stomach, respectively. The enzyme activity had no obvious change after incubation in in vitro simulated intestine for 120 min. This result shows that gkdA has a superior tolerance to gastrointestinal fluids than do simple thermophilic enzymes, meaning that the enzyme is not inactivated when in the stomach and intestines. Compared with simple thermophilic enzymes, this enzyme is more promising for use in the feed industry.

Conclusions

We cloned, identified and characterized the putative protein (gkdA) from Bacillus licheniformis SR01. Structure analysis and function identification showed that this interesting glycosidase is multifunctional and has a structure similar to glucanase. Characterization studies showed that the enzyme has excellent thermal stability and a better tolerance to a gastrointestinal environment, SDS, and other metal ions. To our knowledge, no multifunctional glycosidase has these superior enzyme properties, especially its heat resistance and versatility. These excellent properties make the enzyme a promising candidate for a new feed enzyme additive, which could remove the difficult regulation coordination problems of existing complex enzymes. To obtain more information on the enzyme, the crystal structure, catalytic mechanism, and thermal stability mechanism, should be further studied. We believe that gkdA will be an efficient multifunctional glycosidase for use in the feed, food, medicine, and other industries.

Acknowledgments

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