Properties of a Metagenome-Derived $\beta$-Glucosidase from the Contents of Rabbit Cecum

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In this study, a previously cloned $\beta$-glucosidase gene, umbgl3B, was heterologically expressed in Escherichia coli, and the biochemical properties of the purified enzyme were characterized. The recombinant enzyme was stable over a wide range of pH values (5.0–9.0) and below 30°C. It displayed optimum enzymatic activity at pH 6.5 at 40°C, under condition similar to that in the rabbit cecum, suggesting an active role of the native enzyme in vivo. The recombinant $\beta$-glucosidase Umbgl3B showed high activity to aryl-$\beta$-O-glycosides and low activity to cellooligosaccharides, with a polymerization degree of less than 5. The enzyme had no activity toward long cellooligosaccharides or polysaccharides. The aspartic acid residue, D772, of the wild-type Umbgl3B was predicted as a nucleophile. Mutant D772A was constructed. It showed less than 1/10,000 activity of the wild-type enzyme, but had the same properties, suggesting that residue D772 plays a key role in the enzyme’s activity.

Key words: $\beta$-glucosidase; expression; characterization; mutagenesis; rabbit cecum

$\beta$-Glucosidase (EC 3.2.1.21) hydrolyzes the $\beta$-glucosidic linkages of glucosides, such as cellobiose, cellooligosaccharides, plant-derived $\beta$-glucosides and other chemically related $\beta$-glucosides.1,2 It is one of the three enzymes in the complete cellulase system composed of $\beta$-1,4-endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and $\beta$-glucosidase. While endoglucanase and exoglucanase act together to solubilize crystalline cellulose by digesting it into soluble sugars, $\beta$-glucosidase hydrolyzes cellobiose and cellooligosaccharides into glucose. Additionally, $\beta$-glucosidase stimulates the rate and extent of cellulose hydrolysis by eliminating cellobiose inhibition on endo- and exo-glucanase activities.3,4 Thus $\beta$-glucosidase plays an important role in the utilization of biomass. It has also attracted considerable attention in the food industry for its biotechnological applications, such as enhancing the aromas of fruit juice and tea, removing bitter components of citrus products, and detoxification of cassava.5,6 $\beta$-Glucosidases are commonly present in both eukaryotic and prokaryotic organisms. The digestive tracts of herbivores harbor symbiotic microorganisms to help the host with the digestion of cellulose feeds by producing complete sets of cellulases. Many $\beta$-glucosidases were isolated and characterized from cultured bacteria7–9 and fungi10 in the rumens. Two $\beta$-glucosidase genes have been identified from uncultured samples in the cow rumen11 and the large-bowel of mice.12 However, the biochemical characterization of these metagenome-derived $\beta$-glucosidases has not been reported. We recently constructed a cosmid library in Escherichia coli with metagenomic DNA isolated directly from the contents of the rabbit cecum. Seven $\beta$-glucosidase genes and four $\beta$-1,4-endoglucanase genes were identified from the metagenomic library, which contained about 32,500 clones and had a capacity of about 1.14 × 109 bp.8 Among these $\beta$-glucosidase genes, umbgl3B (accession no. DQ182493) encoded a glycosyl hydrolase family 3 (GHF 3) $\beta$-glucosidase that displayed the highest activities when assayed on substrate-embedded agar plates and by direct enzymatic activity assay with cytoplasmatic extracts. To understand better the biochemical properties of enzyme Umbgl3B, in this study, we selected this gene for heterologous expression in a bacterial system and further characterization of its product.

Materials and Methods

Expression of umbgl3B and purification of recombinant Umbgl3B.

The complete open reading frame of umbgl3B was amplified by polymerase chain reaction (PCR) from the plasmid DNA, which contained the original umbgl3B,14 with forward primer 5’-GAC-GGATCCGAGAAGATTCTTCATCCCCTCTGATG-3’ (umbgl3B-F), and reverse primer 5’-GTCGACGATCCGAGCCTCCGTGTCGCA-CACCAAC-3’ (umbgl3B-R), each containing a BamHI site at the 5’ end (underlined). PCR was carried out for 30 cycles of 30 s at 94°C, 30 s at 43°C, and 3 min at 72°C, with a final 10 min extension at 72°C. The PCR product was cloned into expression vector pET-30a(+) (Novagen, Madison, WI), and the construct was transformed into expression host E. coli BL21 (DE3) pLyS8 (Novagen, Madison, WI). The bacterial transformants were screened first by activity assay on Luria-Bertani (LB) agar plates embedded with 0.1% esculin hydrate.
Characterization of a Novel β-Glucosidase

Site-directed mutagenesis of umbgl3B. The previously predicted nucleophile of Umg3B, the aspartic acid residue D772,19 was replaced by an alanine through site-directed mutagenesis. The gene encoding the mutant enzyme (D772A) was obtained by recombinant PCR with primers designed to change the D772 codon (GAC) into an Ala codon (GCC). The primers used to amplify the upstream fragment were umbgl3B-F and 5′-ACGTGAAACGGCGGTCATGAT-3′, and those for the downstream fragment were 5′-ATCATGACGGCCTGCTTTACAC-3′ and umbgl3B-R. The mutant gene was sequenced and compared with wild-type umbgl3B to verify the correct mutation.

Mutant enzyme D772A was expressed and purified in the same way as recombinant Umg3B. Purified recombinant Umg3B and D772A were subjected to SDS–PAGE. Before staining with Coomassie Brilliant Blue G250, the gel was soaked and washed 3 times with a refolding buffer (10 mM potassium phosphate buffer at pH 7.0) to dilute SDS and allow renaturation of the denatured proteins, as described by Ali et al.,16 then stained with 0.1% citrate-phosphate buffer (pH 6.0) containing 0.1% esculin hydrate and 0.2% ferric ammonium citrate to visualize the activities on the gel. The activity of D772A toward p-NP was measured under routine conditions at a protein concentration of 2.0 mg/mL. pH and temperature profiles of D772A activity were also studied as for recombinant Umg3B.

Results and Discussion

Expression of umbgl3B and purification of recombinant Umg3B

The bacterial transformants were screened by assays on substrate-embedded LB agar plates and PCR to confirm the presence of the umbgl3B gene (data not shown). SDS–PAGE analysis detected a specific protein band from crude extracts of the expression cells that were induced by IPTG (Fig. 1, lane 3), but not in cells without IPTG treatment (Fig. 1, lane 2). The expressed recombinant Umg3B was estimated to have a molecular weight of about 100 kDa on the denatured gel.

0.2% ferric ammonium citrate, and 0.5 mM isopropyl D-thiogalactopyranoside (IPTG), with a supplement of 25 μg/mL of kanamycin and 34 μg/mL of chloramphenicol, then by PCR to select the positive clones that harbored the target gene.

The positive bacterial clone was cultivated in 150 mL of LB medium containing 25 μg/mL of kanamycin and 34 μg/mL of chloramphenicol at 37 °C, and protein expression was induced with 0.5 mM IPTG. Cells were harvested by centrifugation, resuspended in 2.5 mL of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, with pH adjusted to 8.0 with NaOH), and sonicated. After centrifugation, the supernatant was collected as a crude extract of total proteins. Because the recombinant protein was tagged at both the N and C terminals with 6 × His residues, the crude extract was purified first by affinity chromatography using nickel-nitrotriacetic acid agarose resin (Qiagen, Valencia, CA) according to the manufacturer’s manual. Secondary and tertiary chromatography was performed on an AKTA Explorer 100 system (Amersham Biosciences, Uppsala, Sweden) with column Mono-Q 5/50 GL. The column was equilibrated with 50 mM citrate-phosphate buffer at pH 6.0 and eluted with the same buffer containing a linear gradient of 0–1.0 M NaCl at a flow rate of 0.8 mL/min.

Protein and enzyme assays. Protein was quantified by the Bradford method with bovine serum albumin as standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli.

Enzyme activity on p-nitrophenyl β-D-glucopyranoside (p-NPG, Sigma, St. Louis, MO) was determined according to Odoux et al.,11 with a minor modification. The purified enzyme was diluted 20–50 times in 50 mM citrate-phosphate buffer (pH 6.0) to a concentration of 14 μg/mL for the routine enzyme assay, in which 10 μL of the enzyme was added to 130 μL of 0.1 mM citrate-phosphate buffer (pH 6.0) containing 2.5 mM p-NPG. The reaction was carried out at 28 °C for 15 min and was stopped with 70 μL of 0.4 M Na2CO3. The release of p-nitrophenol (p-NP) was measured at 28°C and ended with 400 μL of dinitrosalicylic acid reagent. The released reducing sugars were measured as glucose equivalents at 530 nm, as described by Miller.14 The cellooligosaccharides were hydrolyzed under routine assay conditions with replacement of p-NPG by 2.5 mM of each cellooligosaccharide, an enzyme concentration of 140 μg/mL, and an incubation time of 2 h. The reactions were terminated by boiling, and the glucose generated was detected by the glucose oxidase method.15

To investigate the effects of various metal ions and chelating agents on the enzyme activity, 50 mM NaH2PO4/C12,50 mM NaH2PO4/C2, and 5.0 mM EDTA was used in the routine enzyme assay, as described above.

Characterization of recombinant Umbgl3B. The effect of pH on enzyme activity was assayed at 28 °C, and the effect of temperature on was then assayed at pH 6.0 (0.1 mM citrate-phosphate buffer). pH Stability was studied as described by Eckert and Schneider,21 and thermal stability was evaluated according to the method of Inoue et al.13

p-NPC, p-NPX, CMC, oat spelt xylan, and cellooligosaccharides (including cellobiose, cellotriose, cellotetraose, cellpentaoose, and cellhexaose) were applied to test the substrate specificity of the recombinant Umg3B. All tested substrates were purchased from Sigma. p-NPC (in 2.5 mM) and p-NPX (in 2.5 mM) were assayed routinely as described above with a prolonged reaction time of 2 h. For CMC and xylan, 20 μL of the diluted enzyme was added to 180 μL of 0.1 mM citrate-phosphate buffer (pH 6.0) containing 1% (w/v) CMC or xylan. Reactions were carried out for 2 h at 28 °C and ended with 400 μL of dinitrosalicylic acid reagent. The released reducing sugars were measured as glucose equivalents at 530 nm, as described by Miller.14 The cellooligosaccharides were hydrolyzed under routine assay conditions with replacement of p-NPG by 2.5 mM of each cellooligosaccharide, an enzyme concentration of 140 μg/mL, and an incubation time of 2 h. The reactions were terminated by boiling, and the glucose generated was detected by the glucose oxidase method.15

Fig. 1. SDS–PAGE Analysis of the Expression and Purification of Recombinant Enzyme Umg3B and Mutant Enzyme D772A. Lanes 1 to 6, SDS–PAGE (8.0%) stained with Coomassie Brilliant Blue; lane 1, protein molecular weight marker (no. SM0431, Fermentas, Vilnius, Lithuania); lane 2, crude extracts of uninduced expression cells harboring recombinant umbgl3B; lane 3, crude extracts of Umg3B from expression cells induced by IPTG; lane 4, crude extracts of D772A; lane 5, recombinant Umg3B purified by affinity chromatography and anion-exchange chromatography; lane 6, D772A purified by affinity chromatography and anion-exchange chromatography. Lanes 7 and 8, renatured SDS–PAGE gel treated with esculin solution. Purified Umg3B showed β-glucosidase activity with a dark band on the gel (lane 7), while purified D772A did not (lane 8).
consistent with the calculated molecular size of 101 kDa based on its amino acid sequence. These results indicated expression of the recombinant umbgl3B in E. coli BL21 (DE3) pLysS.

Two-step purification by affinity chromatography and anion-exchange chromatography purified the recombinant Umbgl3B to apparent homogeneity (Fig. 1, lane 5), and increased the specific activity of the enzyme from 2.91 U/mg of the crude extract to 46.3 U/mg after purification. Thus a 16-fold enrichment of the enzyme was achieved.

Characterization of recombinant Umbgl3B

Purified recombinant Umbgl3B hydrolyzed p-NPG efficiently between pH 5.5 and 6.5, and had optimal activity at pH 6.0 when assayed at 28 °C (Fig. 2a). It was stable over a broad range of pHs from 5.0 to 9.0, retaining more than 90% of its activity (Fig. 2b). The enzyme exhibited optimum activity at 40 °C when assayed at pH 6.0 for 15 min (Fig. 2c). However, the thermal stability data showed that the enzyme was stable at temperatures below 30 °C with over 90% of activity remaining, and lost its activity significantly at temperatures above 30 °C (Fig. 2d). The pH-activity profile was also investigated at 40 °C. The enzyme reached its highest activity (1.67-fold higher than at 28 °C) at pH 6.5, which is the physiological condition of the rabbit cecum (pH 6.5 and 39 °C). This result indicates that the native Umbgl3B protein might play an active role of β-glucosidase under in vivo conditions in the rabbit cecum.

β-Glucosidases mainly catalyze scission of the β-1,4-glucosidic linkage in cellobiose, short-chain cellobio-oligosaccharides (degree of polymerization, 3–8), and aryl β-D-glucosides (such as p-NPG). The substrate specificity of recombinant Umbgl3B is shown in Table 1. The enzyme had low activity toward p-NPC, p-NPX, cellobiose, cellotriose, and cellotetraose. The hydrolytic activity of recombinant Umbgl3B on p-NPG was 100 times greater than that on cellobiose, suggesting that recombinant Umbgl3B is specific for aryl β-D-gluco-sides. The hydrolytic activity of recombinant Umbgl3B on cellobio-oligosaccharides became weaker as the degree of polymerization rose, and Umbgl3B did not hydrolyze long-chain cellobio-oligosaccharides such as cellopentaose and cellohexaose. No hydrolysis of polysaccharides such as CMC and xylan by the recombinant Umbgl3B was detected (Table 1).

The presence of CoCl₂ and CaCl₂ significantly increased enzyme activities, to 129% and 124% of the baseline level respectively. MnCl₂, FeCl₂, and MgCl₂ also increased enzyme activities to some degree, while CuCl₂ and CrCl₂ inhibited enzyme activity to 86% and 42% respectively. Chelating agent EDTA was an effective inhibitor, dramatically reducing enzyme activity, to 32% of the baseline level (Table 2).
Site-directed mutagenesis of umbgl3B

β-Glucosidases are classified into GHF 1 or GHF 3 based on the similarities of their amino acid sequences and catalysis mechanisms. Two catalytic residues are known to be involved in the catalytic function of the GHF 3 β-glucosidases. A conserved catalytic Asp residue works as the nucleophile, attacking the substrate anomeric center to form a covalent glucosyl-enzyme intermediate. The other catalytic residue, a Glu or a His, might act as a proton donor, but this needs to be confirmed.17–19) D28520) and D24717) of β-glucosidases from barley and Flavobacterium meningosepticum have been identified to be the nucleophiles by structure and mutagenesis studies respectively.

Residue D772 of wild-type Umbgl3B was predicted to be a nucleophile by multiple sequence alignment.5) Site-directed mutagenesis was conducted to study the active site of Umbgl3B. A sequence comparison of the wild-type and mutant umbgl3B genes showed that only one base of umbgl3B had been changed in the mutant at the expected site, with the codon encoding D772 replaced by the codon encoding A772. The mutant enzyme was overexpressed in the E. coli host (Fig. 1, lane 4), and was also purified to apparent homogeneity (Fig. 1, lane 6). The cells expressing the mutant D772A showed no activity on plate assay. On the renaturation gel, purified recombinant Umbgl3B showed activity upon refolding (Fig. 1, lane 7), whereas no activity of D772A was observed (Fig. 1, lane 8).

However, hydrolysis of p-NPG by D772A was still detectable in enzyme assay, with the concentration of the protein increased to as high as 2.0 mg/ml, even though the specific activity was negligible (3.15 × 10⁻³ U/mg). D772A presented pH and temperature profiles on enzyme activity similar to those of recombinant Umbgl3B, except for low activity (Fig. 2a and c). The mutant enzyme D772A also showed optimal pH and temperature at 6.5 and 40 °C. In the study of Li et al.,17) mutation of D247 led to 30,000- to 200,000-fold lower activity of the wild-type β-glucosidase from Flavobacterium meningosepticum. Mutant D772A retained less than 1/10,000 activity of wild-type Umbgl3B, indicating that D772 is a key residue for the enzyme’s activity.

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References