Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–7, 2017

www.elsevier.com/locate/jbiosc

Carbohydrate-binding module assisted purification and immobilization of β-glucosidase onto cellulose and application in hydrolysis of soybean isoflavone glycosides

Fei Chang,[1,2,3] Saisai Xue,[1,2,3] Xiaqing Xie,[1] Wei Fang,[1,2,3] Zemin Fang,[1,2,3,*] and Yazhong Xiao[1,2,3]

School of Life Sciences, Anhui University, Hefei, Anhui 230601, China,[1] Anhui Key Laboratory of Modern Biomanufacturing, Hefei, Anhui 230601, China,[2] and Anhui Provincial Engineering Technology Research Center of Microorganisms and Biocatalysis, Hefei, Anhui 230601, China[3]

Received 10 July 2017; accepted 7 September 2017
Available online xxx

Complicated purification steps, together with the fact that β-glucosidase has to be tolerant to ethanol restricts the application of β-glucosidase in isoflavone aglycone hydrolyzing process. β-Glucosidase Bgl1A(A24S/F297Y) is a promising enzyme in hydrolyzing isoflavones. In this work, six different carbohydrate-binding modules (CBMs), which were from 3 families, were fused to the C-terminal of Bgl1A(A24S/F297Y), respectively, to simplify the enzyme preparation process. The fusion proteins were expressed in *Escherichia coli* and adsorbed onto cellulose. The Bgl-CBM_{24} was found to have the highest immobilization efficiency at room temperature within 1 h adsorption. Notably, 1-g cellulose absorbs up to 254.9 ± 5.7 U of Bgl-CBM_{24}. Interestingly, the immobilized Bgl-CBM_{24} showed improved ethanol tolerance ability, with the IC_{50} of 35% (v/v) ethanol. Bgl-CBM_{24} effectively hydrolyze soybean isoflavone glycosides. The hydrolysis rate of daidzin and gemistin was 85.22 ± 3.24% and 82.14 ± 3.82% within 10 min, with the concentrations of daidzein and genistein increased by 6.36 ± 0.18 mM and 3.98 ± 0.22 mM, respectively. In the repetitive hydrolytic cycles, the concentrations of daidzein and genistein still increased by 3.07 ± 0.24 mM and 1.94 ± 0.34 mM in the fourth cycle with 20% (v/v) ethanol. These results suggest that the immobilized Bgl-CBM_{24} has excellent potential in the preparation of isoflavone aglycones.

[**Key words:** β-Glucosidases; Immobilization; Carbohydrate-binding module; Cellulose; Isoflavone aglycones]

Isoflavones, a class of nonsteroidal estrogens, are often termed as phytoestrogens, because they bind to the estrogen receptors. Recent studies have shown that isoflavones have excellent nutritional properties and health-promoting characteristics (1,2). For example, isoflavones have a protective effect against hot flashes and can ease symptoms of postmenopausal women (3,4). Evidence has also suggested that supplementation with the dietary phytoestrogen genistein may reduce the risk of osteoporosis without any associated side effects (5). It has also been suggested that dietary isoflavones intake has antimutagenic effects and protective role against cardiovascular disease in postmenopausal women (6,7).

Soybean is rich in isoflavones that exist mainly in the form of glycosides. However, it has been proven that the pharmacological effects of isoflavones are not directly correlated to glycosides but isoflavone aglycones, such as daidzein and genistein. This is partially attributed to the fact that isoflavone aglycones could be absorbed faster and more efficient than glycosides in human intestines (8). Thus, in order to improve bioavailability and pharmacological effects, isoflavone glycosides need to be hydrolyzed to aglycones in food industry. Although various strategies such as physical, chemical and enzymatic are available to convert isoflavone glycosides into aglycones, the hydrolysis of isoflavones with β-glucosidases is the most attractive because of its high efficiency and operational safety (9).

β-Glucosidases (β-D-glucopyranoside glucohydrolase; EC 3.2.1.21) are a heterogeneous group of enzymes that hydrolyze β-1,4-glycosidic bond in disaccharides, oligosaccharides, aryl-, and alkyl β-glucosides, and release non-reducing terminal glucosyl residues (10,11). These widespread enzymes have potential applications in a variety of biological processes such as production of ethanol from agricultural wastes and synthesis of useful β-glucosides (12–14). Many studies have demonstrated the possibility of using β-glucosidases in the hydrolysis of isoflavones to aglycones (9,15). However, the complicated purification steps of β-glucosidases make the large-scale enzymatic production of isoflavone aglycones economically unpractical (16,17). Furthermore, high concentration of ethanol (>10%) has to be added into the hydrolysis system in β-glucosidases catalyzed hydrolysis due to the low solubility of isoflavones (18–20), indicating that β-glucosidases have unique properties such as tolerance to ethanol that allows the application of these proteins in other biotechnological processes.

Fusion the target protein with an affinity tag may simplify the purification steps, and help improving the biochemical properties of the target protein. Recently, affinity tags such as carbohydrate-binding module (CBM) have been proven useful to purify and immobilize proteins in one step. A CBM is defined as a contiguous amino acid sequence in a carbohydrate-active enzyme, with a discreet fold having carbohydrate-binding activity (21,22). The use of the biospecific affinity of CBMs showed great advantages: the

* Corresponding author at: School of Life Sciences, Anhui University, Hefei, Anhui 230601, China. Tel./fax: +86 551 63861063.
E-mail address: zemin_fang@ahu.edu.cn (Z. Fang).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.09.001

cellulose is abundant, inexpensive and suitable for the production of food and drugs, the binding is spontaneous without the need of using additional chemical reagents. Furthermore, binding capacity of cellulose is relatively strong with high adsorption rate (22,23). The CBM fusion strategy has been successfully applied in the immobilization of several enzymes such as γ-lactamase and carboxymethyl cellulose (24,25). However, few studies have evaluated the effects of fusion of a CBM at the terminal of a β-glucosidase.

Bgl1A(A24S/F297Y) is a double-site variant of β-glucosidase Bgl1A (GenBank accession no. GU647096) (26), and obtained by replacing residues A24 and F297 with S and Y, respectively, based on site-directed mutagenesis (19). The mutant exhibits excellent ethanol and glucose tolerance, and has good pH- and thermostability. It displays high hydrolysis rates for isoflavone glycosides within a short reaction time (19). In this study, in order to reduce the costs of making isoflavone aglycones using Bgl1A(A24S/F297Y), the CBM fusion strategy has been applied in the immobilization of Bgl1A(A24S/F297Y). Six CBMs were codon optimized according to the codon preference of *Escherichia coli* and fused into the C-terminal of Bgl1A(A24S/F297Y) with a flexible linker GSAGSA. The fusion enzymes were easily purified using cellulose through a one-step centrifugation. Interestingly, immobilized Bgl1A(A24S/F297Y) showed an enhanced tolerance to ethanol. Most importantly, immobilized Bgl1A(A24S/F297Y) showed high efficiency and productivity in hydrolyzing soybean isoflavones.

MATERIALS AND METHODS

Strains, plasmids, reagents and medium The strains and plasmids used in this study are listed in Table S1. *E. coli* Trans5α (TransGen, Beijing, China) was used as the genetic manipulation host. *E. coli* BL21(DE3) (TransGen, Beijing, China) was used as the heterologous expression host. Plasmid pET-22b-T7-*bgl*, derived from pET22b(+) (Novagen, Madison, WI, USA) by replacement *bgl1A(A24S/F297Y)* fragment into the downstream of T7 lac promoter, was used for the construction of expression plasmids. Ampicillin, chloramphenicol, and IPTG were obtained from Sangon Biotech (Shanghai, China). pNPG was purchased from Sigma−Aldrich (St. Louis, MO, USA). The insoluble microcrystalline cellulose with an average particle size of 25 μm was acquired from Aladdin Chemistry (Shanghai, China). Ni^{2+}-charged chelating sepharose fast flow was purchased from GE Healthcare (Uppsala, Sweden). Soy isoflavone powder (Sinosoy40) was purchased from Jinmen Deai Biological Engineering (Jinmen, China). All other chemicals were of analytical grade unless otherwise specified. Lysogeny broth (per liter contains 5 g yeast extract, 10 g tryptone, 10 g NaCl) was used as culture medium in plasmid construction. Standard TB medium (per liter contained 4 g glycerol, 24 g yeast extract, 12 g peptone, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) was used as culture medium in 250-mL Erlenmeyer flasks.

Plasmid construction and fusion protein expression The primers used in this study are listed in Table S2. Six CBM sequences from different species, named as CBM_3 (*Tramates* sp. AH28-2, GenBank accession no. KQ464076.1), CBM_{24} (*Clostridium thermocellum*, HF912724.1), CBM_{60} (*Clostridium cellulovorans* 743B, CP002160.1), CBM_{67} (*Ruminiclostridium thermocellum*, CCV01467.1), CBM_{91} (*Cellulosilyticum ruminicola* JCM 14822, ACZ98591.1) and CBM_{92} (*R. thermocellum*, AAA20892.1), were chosen in order to use cellulose as a substrate to immobilize and purify the enzyme. The CBM genes were codon optimized according to the codon preference of *E. coli* and synthesized by Sangong Biotech (Shanghai, China). Then the CBMs were fused to the C-terminal of Bgl1A(A24S/F297Y) with a flexible linker GSAGSA using overlap extension-PCR. After digestion with *Nde* I and *Xho* I, The bgl-CBM sequences were inserted downstream of T7 promoter of plasmid pET22b(+). The plasmids were transformed into *E. coli* BL21(DE3) and used for fusion proteins expression.

In order to induce the expression of fusion proteins, the expression strains were cultivated at 28°C in 50 mL TB medium supplemented with 100 μg/mL ampicillin in an orbital shaker. Induction was started when the cell density (OD_{600}) reached 0.6, IPTG was added at a final concentration of 0.75 mM as described previously (19). *E. coli* BL21(DE3) containing pET22b-T7-*bgl* was used as the control.

Immobilization of fusion proteins onto cellulose The culture was centrifuged at 15,000 ×*g* for 5 min to collect cells. The cells were resuspended in Na_2HPO_4-citric acid buffer (pH 6.5) and disrupted by sonication, and then centrifuged at 30,000 ×*g* and 4°C for 30 min. The cell-free supernatant (crude enzyme) was collected, and NaCl was supplemented at a final concentration of 200 mM. The crude enzyme was diluted to 10 U/mL of β-glucosidase activity. Finally, 0.5 g cellulose was added into 10 mL of crude enzyme solution. The mixture was incubated at 25°C under mild shaking followed by centrifugation at 4000 ×*g* for 5 min. The cellulose bound fusion proteins were in sediment and were washed 3 times with Na_2HPO_4-citric acid buffer (50 mM, pH 6.5) to remove the unbound or loosely bound protein. The immobilization efficiency was calculated by measuring the β-glucosidase activity in supernatant and cellulose suspension.

β-Glucosidase activity assay β-Glucosidase activity was tested using *p*NPG as substrate. The assay mixture consists of 25 μL appropriately diluted enzyme sample and 475 μL 50 mM Na_2HPO_4-citric acid buffer (pH 6.5), containing 5 mM *p*NPG. Enzyme activity was determined by monitoring absorption changes at 405 nm. A unit of enzyme was defined as the amount of β-glucosidase required for releasing 1 μmol of *p*NP per minute. Reactions with heat-treated samples were used as controls. All results were the average of data from triplicate experiments.

Biochemical characterization and kinetic parameters of the immobilized enzymes The effect of pH on the activity of immobilized Bgl-CBMs was determined in 50 mM Na_2HPO_4-citric acid buffer with pHs ranging from 5.5 to 8.5. The effect of temperature was assayed at 20−55°C in 50 mM Na_2HPO_4-citric acid buffer (pH 6.5). The effect of ethanol on the activity of immobilized Bgl-CBMs was determined in 50 mM Na_2HPO_4-citric acid buffer (pH 6.5) with different concentrations of ethanol (0−50%, v/v). The stability of the immobilized Bgl-CBMs in the presence of ethanol was assessed by incubating appropriate volumes of the immobilized enzyme with different concentrations of ethanol (5−20%, v/v) in tightly closed 5 mL microtubes at 30°C. Aliquots were taken at intervals of 10 min, and the residual activity was determined using *p*NPG as substrate. Enzyme activity in the absence of ethanol was used as control.

To determine the kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) of the enzyme, the purified Bgl1A(A24S/F297Y) and immobilized Bgl-CBMs were assessed at the optimal conditions using 0.05−5.0 mM *p*NPG as substrate. The kinetic constants were calculated by fitting the experimental data to the Michaelis-Menten equation using Origin 8.0. The catalytic constant, k_{cat} value, is equal to V_{max} divided by the total enzyme molar concentration calculated by using the molecular weight of the enzyme. Bgl1A(A24S/F297Y) with C-terminal His_6 purified by Ni^{2+} charged Chelating Sepharose Fast Flow (GE Healthcare, Uppsala, Sweden) was used as control.

Hydrolytic property of the immobilized enzymes To test the reactivity of immobilized enzymes towards high concentrations of isoflavones, soy isoflavone powder extract was used as substrate. The extraction of soybean isoflavone from powder was performed according to the method reported by Fang et al. (19). Hydrolysis of isoflavone glycosides by the immobilized enzyme was determined in 500 μL of 50 mM Na_2HPO_4-citrate buffer (pH 6.5) containing an appropriate volume of isoflavone extract and the immobilized enzymes.

Effects of isoflavone flour extract [5−20% (v/v)] on the hydrolysis rate were evaluated in 500 μL reaction buffer at 30°C for 30 min to determine the optimal reaction condition. Samples were taken every 10 min and the conversion efficiency was determined. To evaluate the reusability of the immobilized enzyme, 8.3%, 16.6% and 33.2% (v/v) volume of isoflavones flour extract, (5%, 10% and 20% (v/v) ethanol introduced into the hydrolysis reaction mixture) were added to the reaction mixture. The hydrolyzed solutions were added into 4.5 mL of 60% (v/v) ethanol to stop the reaction. The mixture was centrifuged at 16,000 ×*g* for 20 min, and the supernatant was filtered through a 0.22 μm filter. Samples (20 μL) were withdrawn and analyzed by HPLC equipped with Agilent Eclipse Plus C18 column (Agilent Corp., Palo Alto, CA, USA) with a UV detector set at 254 nm. A linear HPLC gradient was employed, using solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetoni-trile) at 25°C. Solvent B was gradually increased from 5% to 100% over a time span of 60 min at a flow rate of 0.5 mL/min. Peaks of soy isoflavone glucosides (daidzin and genistin) and the corresponding aglycons were identified by matching the retention times with the commercial standards (Sigma Chemical Co., USA). The hydrolysis rate was calculated using the following equation (27):

$$\text{Hydrolysis rate (\%)} = 100 \times (\text{isoflavone glycosides in control samples} - \text{isoflavone glycosides in hydrolyzed samples}) / \text{isoflavoneglycosides in control samples} \quad (1)$$

Determination of cell density Cell growth was monitored during cultivation by measuring OD_{600} using a spectrophotometer. Samples were appropriately diluted with 0.9% (w/v) NaCl before determination. Cells were withdrawn at different time intervals and were collected by centrifugation. The pellets were washed and resuspended in 50 mM Na_2HPO_4-citric acid buffer (pH 6.5) and disrupted by sonication. The cell lysates were centrifuged to separate the soluble and insoluble fraction. The supernatants were used to detect the enzyme activity.

RESULTS

Expression of β-glucosidase fusion proteins The β-glucosidase structural gene and the linker sequence were 1326-bp and 18-bp fragment. The six CBM encoding genes were 180-bp

Please cite this article in press as: Chang, F., et al., Carbohydrate-binding module assisted purification and immobilization of β-glucosidase onto cellulose and application in hydrolysis of soybean isoflavone glycosides, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.09.001