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An extremely thermostable maltogenic amylase from *Staphylothermus marinus*: *Bacillus* expression of the gene and its application in genistin glycosylation

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ABSTRACT

The most extremely thermostable maltogenic amylase (SMMA) from archaeon *Staphylothermus marinus* has many potential applications in food processing. To ensure safety of microbial origin, a recombinant plasmid containing the enzymic gene and a constitutive promoter AmyR2 was constructed, and then transformed into a GRAS microorganism *Bacillus subtilis*. The purified SMMA from the liquid cultures of *Bacillus* has a specific activity of 66.96 U/mg, two times more than that from *Escherichia coli*. SMMA was further employed to catalyze the genistion glycosylation using γ-CD as both glucosyl donors and solubilizer. Glycosylated genistins with one to four additional α-glucosyls and a molar percentage of 69.87% in genistin reaction mixture were identified and quantified by HPLC–UV–MS. The glycosylated genistins at 0.2–1.2 mM showed an enhanced DPPH free radical scavenging capacity. To our knowledge, this is the first report on the *Bacillus* expression of archaeal maltogenic amylase.

1. Introduction

Maltogenic amylase (EC 3.2.1.133), which naturally occurs in many microorganisms, plays the critical roles in the degradation of maltodextrin and cyclodextrin (CD) in carbohydrate metabolism [1,2].

In vitro activities, these enzymes exhibit multi-substrate specificities and various catalytic capabilities of hydrolysis and transglycosylation for cleaving and/or forming α-(1,3)-, α-(1,4)- and α-(1,6)-glucosidic linkages [3,4]. In molecular structure, they possess a central catalytic domain of $(\beta/\alpha)_8$-barrel fold of a typical α-amylase and a special N-terminal starch-binding domain. Maltogenic amylase has been classified into glycoside hydrolases family GH13_20 accordingly [5].

Many maltogenic amylases of different microbial origin have been cloned and characterized for numerous food applications. Among them, *Bacillus stearothermophilus* maltogenic amylase (BSMA) were most widely used in starch and baking industry to produce low amylose starch [6–8] and retard bread staling [9]. To employ the transglycosylation activity, BSMA was also used to glycosylate flavonoids, such as naringin [10] and puerarin [11], to enhance their water solubility.

However, we found that bacterial maltogenic amylases with the optimal catalytic temperature of 30–70 °C failed to glycosylate soybean isoflavone glycosides, possible due to their high hydrophobicity. Particularly, genistin is hardly dissolved in water at room temperature. Its solubility is the lowest compared with those of daidzin and puerarin. The other α-glucanotransferases from glycoside hydrolase family 13, such as cyclodextrin glucanotransferase (EC 2.4.1.19) of alkalophilic *Bacillus* sp. I-5, 4-α-glucanotransferase (EC 2.4.1.25) of *Thermus scotoductus*, could transfer different numbers of glucosyl from starch to the glucosyl of genistin at 55–70 °C in water. But the maximum concentration of genistin in the glycosylation reaction was only 0.3 mg/mL [12]. To improve the glycosylation efficiency of soybean isoflavone glycosides, it is necessary to search thermostable α-glucanotransferase, such as maltogenic amylase, with high transglycosylation activity and high optimal catalytic temperature, as high temperatures often increase the substrate concentration, accelerate the reaction rate and decrease the possibility of microbial contamination.

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Recently, an archaeal maltogenic amylase from the hyperthermophilic *Staphylothermus marinus* (SMMA), was heterologously expressed in *Escherichia coli* in our laboratory [13]. The purified recombinant SMMA was found to be the most extremely thermostable maltogenic amylase till now with an optimal temperature of 100 °C and high hydrolytic activity under mild acidic conditions (pH 4.0–6.0). The crystal structure of SMMA revealed that a N′ domain in a much longer N-terminal region together with the catalytic domain and the C-terminal domain formed the substrate binding pocket that distinguishes the archaeal maltogenic amylase from classic bacterial maltogenic amylases [14,15].

In this research, SMMA was firstly heterologously expressed in the GRAS microorganism *B. subtilis* to ensure the safety of origin for potential applications in foods. Then, the purified SMMA was employed to catalyze genistin glycosylation using γ-CD as both glucosyl donor and solubilizer. Finally, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity of glycosylated genistins was evaluated.

2. Materials and methods

2.1. Chemicals

Genistin, γ-CD, and DPPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was obtained from Thermo Fisher (Pittsburgh, PA, USA). Yeast extract and tryptone were purchased from Oxoid (Basingstoke, Hampshire, England). Restriction enzymes, PrimeSTAR® HS DNA Polymerase, and *B. subtilis* ISW1214 were supplied by Takara (Dalian, China). Shuttle vector pBURTAMY with an AmyR2 promoter was kindly provided by Prof. Young-Wan Kim (Department of Food and Biotechnology, Korea University). Water was prepared using a double distilled water system (BSZ-2, Botonyc, Shanghai, China). All other chemicals used were of reagent grade and were purchased from Sinopharm (Shanghai, China).

2.2. Subcloning into Bacillus vector

The gene encoding SMMA (Smar_0613; Accession: ABN69720.1; GI: 126014342) was amplified from plasmid pSMMA6xH [13] by PCR (C1000 Touch™ Thermal Cycler; Bio-Rad, Hercules, CA, USA) using PrimeSTAR® HS DNA Polymerase (30 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 120 s). The forward (5'-GGCTGTATAGCATATGTACAAAATTATTGG-3') and reverse (5'-TAGCTCGAGTATATTACAAGGTTTAGAACC-3') primers in the reaction contained *Nde*I and *Xho*I restriction sites (underlined), respectively. The amplified fragment (2.1 kb) was digested with these two restriction enzymes, and ligated into the expression vector pBURTAMY [16] digested with the same restriction enzymes. *E. coli* MC1061 was transformed by the ligation solution using $CaCl_2$ method. The plasmid pBURT-Smar0613 was extracted from the LBA [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and ampicillin (100 μg/mL)] liquid culture of a colony on LBA plate [1.5(w/v) agar].

2.3. Enzyme preparation and assays

B. subtilis ISW1214 was transformed by the pBURT-Smar0613 using Dubnau method with a slight modification [17]. The transformants were inoculated into two 250 mL medium A (3.3% tryptone, 2% yeast extract, 0.74% NaCl, 0.8% Na_2HPO_4, 0.4% KH_2PO_4, 2% casamino acids, and 0.06 mmol L^{-1} $MnCl_2$) supplemented with kanamycin (40 μg/mL), and then cultured in an orbital shaker at 250 rpm and 37 °C for 24 h.

The cells were collected by centrifugation at 7000 × *g* and 4 °C for 20 min (Avanti® J-26XP Centrifuge; Beckman Coulter, Brea, CA, USA), resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 5 mM imidazole), and sonicated 5 times for 3 min each for 25 mL each of resuspended *B. subtilis* in an ice bath using an ultrasonic processor with a 1 2⁄ ″ (13 mm) probe with replaceable tip at net power output 750 W, frequency 20 kHz, amplitude 35%, pulse on 2 s and pulse off 1 s (VCX 750; Sonics & Materials, Newtown, CT, USA). The cell-free supernatant (56.5 mL) was then collected by centrifugation at 9000 × *g* and 4 °C for 20 min, and then heated at 70 °C for 15 min to remove all thermolabile proteins. The crude enzyme from heat treatment (53 mL) was further purified by an ÄKTApurifier UPC 10 system with a Ni Sepharose™ 6 Fast Flow column (2.6 × 10 cm) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The active fractions in elution buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 500 mM imidazole) were concentrated, and then dialyzed by ultrafiltration (Amicon stirred cell model 8400; Merck Millipore, Billerica, MA, USA) against 50 mM Tris-HCl buffer (pH 7.4).

The protein concentration was determined using Bradford method with bovine serum albumin as a standard [18]. The purity and molecular weight of the protein were analysed by discontinuous SDS-PAGE [19]. The hydrolytic activity of SMMA was assayed using DNS method [20]. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per minute.

2.4. Glycosylation reaction

Thirty milligram of genistin and 300 mg of γ-CD were mixed with 24 mL of sodium acetate buffer (50 mM, pH 5.0) in a 50 mL Corning® disposable plastic centrifuge tube, and then preheated in a water bath at 90 °C for 15 min. The reaction was initiated at 90 °C by the addition of 6 mL of SMMA (10 U). After the tube was incubated for 1 h, it was cooled to room temperature. A 50 mL Amberlite XAD-16 resin (Rohm and Haas, Philadelphia, PA, USA) and a Sep-Pak Plus C18 cartridge (Waters, Milford, MA, USA), previously activated with EtOH and water, were used in turn to absorb the genistin glycosides in the reaction solution and to remove the remaining malto-oligosaccharides and salt. The solution eluted with EtOH was used in the following experiments.

2.5. HPLC-UV-MS analysis

The genistin and its glycosylated products were analysed by an Agilent 1290 HPLC system (Agilent Technologies, Palo Alto, CA, USA) interfaced to a micrOTOF-Q II™ mass spectrometer (Bruker Daltonics, Billerica, MA). Samples were filtered through 0.45 μm filter units, and then injected into a ZORBAX Eclipse XDB C18 column (150 × 4.6 mM i.d., 3.5 μm particle size; Agilent). A gradient solvent system consisting of solvents A (methanol/water/formic acid, 20:80:0.1, v/v/v) and B (methanol/water/formic acid, 80:20:0.1, v/v/v) at a flow rate of 0.8 mL/min was used. Solvent B was increased linearly from 0 to 100% in 0–30 min. Electrospray ionization at positive mode was performed using nebulizer 0.4 bar, drying gas 4 L/min, and drying temperature 180 °C, capillary 4000 V. UV spectrum was recorded at 254 nm using a PDA detector. Data were collected and calculated using the DataAnalysis (Version 4.0, Brucker Daltonik GmbH, Bremen, Germany).

2.6. Evaluation of the free radical scavenging capacity

The capacity to scavenge the "stable" free radical DPPH was monitored using Blois method [21] with minor modifications. Four hundred sixty microliter of the diluted reaction solution (×5.0–1.0) and 540 μL of DPPH (0.2 mM) in ethanol were mixed vigorously and then kept in a dark place. After 20 min, the absorbance at 517 nm was recorded using a UV–vis spectrophotometer (UV-2550;