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Degradation of konjac glucomannan by *Thermobifida fusca* thermostable β-mannanase from yeast transformant

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ABSTRACT

Native konjac glucomannan was used as the substrate for thermophilic actinomycetes, *Thermobifida fusca* BCRC19214, to produce β-mannanase. The β-mannanase was purified and five internal amino acid sequences were determined by LC-MS/MS. These sequences had high homology with the β-mannanase from *T. fusca* YX. The *tfm* gene which encoded the β-mannanase was cloned, sequenced and heterologous expressed in *Yarrowia lipolytica* P01g expression system. Recombinant heterologous expression resulted in extracellular β-mannanase production at levels as high as 3.16 U/ml in the culture broth within 48 h cultivation. The recombinant β-mannanase from *Y. lipolytica* transformant had superior thermal property. The optimal temperature of the recombinant β-mannanase from *Y. lipolytica* transformant (pYLSC1-*tfm*) was 80 °C. When native konjac glucomannan was incubated with the recombinant β-mannanase from *Y. lipolytica* transformant (pYLSC1-*tfm*) at 50 °C, there was a fast decrease of viscosity happen during the initial phase of reaction. This viscosity reduction was accompanied by an increase of reducing sugars. The surface of konjac glucomannan film became smooth. After 24 h of treatment, the *DPw* of native konjac glucomannan decreased from 6,435,139 to 3089.

1. Introduction

The plant cell wall is mainly composed of complex cellulose, hemicellulose, and lignin. Hemicelluloses, which act as linkers between lignin and cellulose, are polysaccharides in plant cell walls with β-1,4-linked backbones and an equatorial configuration. Hemicelluloses include xyloglucans, xylans, mannans, glucomannans, and β-1,3, β-1,4-glucans [1]. In addition to xylan, mannan is the other major hemicellulose constituent [2]. Most mannans are contained as glucomannan or galactomannan in softwood or bean seed [3].

The glucomannan from *Amorphophallus konjac* Koch is a polysaccharide that is considered a dietary fiber. The polymer backbone contains both (1→4)-β-D-glucopyranose and β-D-mannopyranose, having glucose and mannose units in a molar ratio of 1:1.6 with a low degree of acetyl group sat the C-6 position [4]. The monosaccharide units are acetylated to an extent of 5–10% [5]. Locust bean gum was the first galactomannan that was used both industrially and in food products. Galactomannans are linear polysaccharides based on a β-l,4-mannan backbone to which single D-galactopyranosyl residues are attached via α-l,6 linkages [6].

β-Mannanases (endo β-l,4-mannan mannohydrolase; EC 3.2.1.78) catalyze the endo-type hydrolysis of β-1,4-mannosidic linkages in β-1,4 mannan, glucomannan, and galactomannan, resulting in the production of partially hydrolyzed mannan and mannooligosaccharides. They are useful in several industrial processes such as reduction of viscosity of coffee extracts, biobleaching of softwood Kraft pulp and bio-refining of lignocellulose [7]. β-Mannanases are listed within glycoside hydrolase (GH) families in the carbohydrate-active enzyme database based on sequence similarity [8]. β-Mannanases belong to the families GH5, GH26, and GH113 and display a $(\beta/\alpha)_8$ barrel-shaped protein folding pattern, and these enzymes are responsible for acid–base-assisted catalysis via a double displacement mechanism involving a covalent glycosyl enzyme intermediate [9,10].

Actinomycetes were widespread soil microorganisms that play significant roles in the decomposition of nature biopolymers such as lignocellulose, pectin, chitin, and keratin [11]. In order to produce enzymes for the enzymatic degradation of nature

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biopolymers, seventy thermophilic actinomycetes with potent extracellular lignocellulolytic enzyme activities were isolated from compost samples collected in Taiwan [12–14]. Of the 70 strains of thermophilic actinomycetes, *Thermobifida fusca* BBRC 19214 [15] exhibited the best mannan hydrolytic activity was used in this study.

To reduce the energy cost for cultivation, some thermostable enzyme genes from thermophilic microorganisms had been cloned and heterologous expressed in mesophilic microorganisms [16,17]. *Yarrowia lipolytica* is nonconventional, aerobic, dimorphic yeast can be found in environments containing hydrophobic substrates. It was widely used in the industry for citric acid, peach flavor and single cell protein production [18]. More recently, *Y. lipolytica* has been regarded as a potential host for heterologous expression of proteins due to its capacity to secrete high levels of extracellular enzymes such as protease, lipase, esterase, and amylase [13,19,20]. Recently, the amylase and acetylxylan esterase genes from *T. fusca* were heterologous expressed in *Y. lipolytica*, *P. Pastoris* and *Escherichia coli* [13]. Within these three expression systems, the *Y. lipolytica* expression system showed the highest enzymatic activities.

This study aimed to over-express the thermostable β-mannanase from the thermophilic actinomycete *T. fusca* BBRC 19214 in *Y. lipolytica* expression system. The properties and nucleotide sequence of the enzyme were studied. The potential applications of the enzyme for konjac glucomannan hydrolysis were also investigated.

2. Materials and methods

2.1. Microorganisms

The thermophilic actinomycete, *T. fusca* BCRC 19214, was routinely cultivated in CYC medium (33 g/l Czapek-Dox powder, 2 g/l yeast extract, and 6 g/l casamino acid, pH 7.2) at 50 °C [15]. *Y. lipolytica* P01g (MatA, leu2-270, ura3-302::URA3, xpr2-332, axp-2) and pYLSC1 were obtained from Yeastern Biotech Co., Ltd. (Taipei, Taiwan).

2.2. Materials

Yeast extract, peptone, Czapek-Dox powder, yeast nitrogen base without amino acids, and agar were purchased from BD (Sparks, MD, USA). T4 DNA ligation kit and restriction endonucleases were purchased from Roche (Mannheim, Germany). For PCR, Vio Twin Pack Kits were purchased from Viogene (Sunnyvale, CA, USA). The Sephacyl S-200, S-300, DEAE-Sepharose CL-6B, and HiTrap Q HP columns were purchased by GE Healthcare (Little Chalfont, UK). The SDS-PAGE molecular weight standards and protein assay kits were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Transformation and screening of Y. lipolytica transformant

The recombinant plasmid was linearized with *Not*I and transformed into *Y. lipolytica* P01g by the method of Xuan et al. [21]. The transformants were selected on YNB agar plates (6.7 g/l yeast nitrogen base without amino acid, 20 g/l glucose) at 28 °C for 2–4 days. The selected transformants were tested for β-mannanase activity. Transformants with high and stable β-mannanase activities were selected for further experiments.

2.4. Biomass

Biomass (mg/ml) was measured by dry cell weight estimation involving filtration of broth samples through predried Whatman® qualitative filter paper, grade 2 (Sigma–Aldrich, MO, USA). The biomass was then washed, dried, cooled in a desiccator, and weighed [12].

2.5. Enzyme purification

All purification procedures were performed at 4 °C in 20 mM sodium phosphate buffer (pH 8.0) unless otherwise stated. After cultivation of *T. fusca* BCRC 19214 or *Y. lipolytica* transformant in the Hinton flask, the fermentation broth was centrifuged (10,000 × *g*, 30 min) to remove the cell pellet. The supernatant was subsequently concentrated by ultrafiltration (Pellicon XL, Biomax 10K, Millipore). The sample was pumped through the membrane module of Pellicon XL with controlled flow rate. The trans-membrane pressure was controlled by the retentate valve. The permeate flow was measured until it was constant. The retentate were collected during filtration process [22].

The concentrate solution was applied to a DEAE-Sepharose CL-6B column (2.6 cm × 10 cm) previously equilibrated with the same buffer. After it was washed with the same buffer to remove inactive protein, the enzyme was eluted with a linear gradient of the same buffer containing NaCl from 0.0 M to 0.5 M. The active fraction eluted were collected and applied to a Sephacryl S-200 column (1.6 cm × 100 cm) previously equilibrated with the same buffer. The eluted enzymatically active fractions were collected and used as the purified enzyme. All of the manipulations were performed following the manufacturer's instructions.

2.6. β-Mannanase activity

The β-mannanase activity was determined in the reaction mixture containing 0.1 ml of appropriately amount of enzyme and 0.9 ml of 20 mM sodium phosphate buffer (pH 8.0) containing 0.5% (w/v) of locust bean gum at 50 °C. The reducing sugar liberated by hydrolysis of locust bean gum in the mixture was determined through the dinitrosalicylic acid method. D-Mannose was used to construct a standard curve. One unit of β-mannanase activity was defined as the amount of enzyme that liberates 1 μmole of mannose per min at 50 °C.

2.7. pH and temperature effects on enzyme activity

To determine the pH-activity profile of the enzyme, the enzyme was diluted in buffer with a pH value that ranged from 4.0 to 11.0. To assess the effects of temperature on enzyme activity, the assay temperature was varied from 40 °C to 90 °C. Thermal stability was assessed by measuring the remaining activity of enzyme after incubation at 40–80 °C over a period of 240 min.

2.8. DNA manipulations

The buffers and reaction conditions used in the present study for the restriction endonucleases, T4 DNA ligase, and PCR were those recommended by the commercial sources. Isolation of DNA fragments, preparation of plasmid DNA, and other standard recombinant DNA techniques were performed [23].

2.9. Determination of the internal amino acid sequence of the purified enzyme by LC-MS/MS

The internal amino acid sequence of β-mannanase was determined by the in-gel digestion of the protein and the sequencing of the different peptides by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Applied Biosystems QStar LC-MS/MS spectrometer (Life Technologies Corp., Carlsbad, USA) as described previously [15].